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ENDOCRINE AND PARACRINE FACTORS RELATED TO PREGNANCY AND INFERTILITY

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ENDOCRINE AND PARACRINE FACTORS RELATED TO PREGNANCY AND INFERTILITY

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Knowledge is the foundation of medicine but so is compassion, one cannot exist without the other

To Michelle, Melanie, Melodi and Filip,

ABSTRACT

Unexplained infertility, one of the common gynecological disorders, affecting 10 to 15 % of women worldwide. Recurrent pregnancy loss affects approximately 1 to 3 % of pregnant women with an unknown etiology in 50 % of cases. Endocrine dysfunctions, including thyroid dysfunction, are one of the related factors to female reproductive disturbances. The aim of the thesis was to explore the importance of thyroid function and endometrial factors for implantation and pregnancy.

Three groups of pregnant women, one group with high risk (n = 88) was compared to a low-risk (n = 511) and a general screening group (n = 699) to study the incidence of subclinical hypothyroidism and hypothyroidism, defined as TSH < 2.5 mIU/L. Women with recurrent pregnancy loss, (n = 165), and controls, (n = 289), were included in genetic analysis and gene expression array of endometrium, (n = 4 and n = 5 respectively). Infertile women (n = 19) and fertile women were included to study thyroid-related proteins in endometrium (n = 28) and Fallopian tube (n = 13). Human embryos, (n= 36), were used to study the effect of thyroid hormone on embryo development.

Thyroid stimulating hormone (TSH), free thyroid hormone (fT4) and TPO antibodies (TPO-Ab) levels were analyzed by use of immunological methods. Genetic variations in the *HABP2* gene was performed by use of TaqMan SNP Genotyping Assays. Gene expression array was used to study mRNA in endometrium of women with recurrent miscarriage at the time of implantation. Immunohistochemistry was used to analyze the presence and distribution of thyroid related proteins in endometrium. Additionally, influence of thyroid hormone (T4) on the early embryo development was analyzed.

Approximately 10 % of all pregnant women, regardless of risk, had elevated TSH levels. Furthermore, hypothyroid women on levothyroxine supplementation had in almost 50 % of cases inadequate treatment. There were no significant differences in the presence of polymorphism in *HABP2* genes in comparison to fertile controls. In total, 124 genes were differently expressed in women with recurrent miscarriage mainly related to immunological processes, particularly shown by upregulation of IL8 in women with recurrent miscarriage. The infertile women showed lower protein staining of TRα1 and MCT8 in endometrium compared to the fertile women. Staining of thyroid related proteins was observed in all different parts of Fallopian tube. T4 supplementation showed improvement of blastocyst development in T4 added media.

In conclusion, general screening for thyroid dysfunction during first trimester of pregnancy is needed to find all women in need to LT4 supplementation. Inflammatory events, especially IL8, might offer a clue to recurrent pregnancy loss while variations in the *HABP2* gene does not seem to be associated with recurrent miscarriage. The expression and distribution of thyroid-related factors in endometrium seem to be related to unexplained infertility. The presence of thyroid-related proteins in fallopian tube and the improvement of embryo development after T4 treatment suggest that a functional thyroid system is important form achievement of a normal pregnancy.

Keywords: TSH screening, pregnancy, Genotype, Hyaluronan–binding protein2 (HABP2), endometrial receptivity, unexplained recurrent miscarriage , thyroid hormone, Fallopian tube, embryo culture, unexplained infertility, MCT8, DIO2, thyroid hormone receptor, TSH receptor.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-IV:

I. **Frida Hosseini Akram**, Bengt Johansson, Gunnar Möllerström, Britt-Marie Landgren, Anneli Stavreus-Evers, Lottie Skjöldebrand Sparre **Incidence of subclinical hypothyroidism and hypothyroidism in early Pregnancy** *Journal of Women's health, 2017, 26, 1231 – 123*

II. **Frida Hosseini-Akram**, Sally Haroun, Signe Altmäe, Lottie Skjöldebrand-Sparre, Helena Åkerud, Inger Sundström Poromaa, Britt-Marie Landgren, Anneli Stavreus-Evers **Hyaluronan-binding protein 2 (HABP2) gene variation in women with recurrent miscarriage** *BMC Women's Health (2018) 18:143*

III: **Frida Hosseini**, Theodora Kunovac Kallak, Helena ÅkerudMD, Britt-Marie Landgren, Lottie Skjöldebrand-Sparre, Anneli Stavreus-Evers **Endometrial gene expression in women with recurrent pregnancy loss reveals up-regulation of the IL8 pathway** *Manuscript, submitted for publication*

IV: **Frida Hosseini Akram**, Lottie Skjöldebrand-Sparre, Britt-Marie Landgren, Fatma Gulen Yaldir, Lusine Aghajanova, Kjell Wånggren, Anneli Stavreus-Evers **Elucidating the role of thyroid hormone receptors in endometrium, fallopian tubes and blastocysts in women with unexplained infertility and healthy fertile controls** *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AITD	Autoimmune thyroid diseases
DIO	iodothyronine deiodinases
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropine
ICSI	Intracytoplasmic sperm injection
IUGR	Intrauterine Growth Retardation
IVF	In vitro fertilization
MCT8	Mono carboxylate transporter-8
SCH	Subclinical hypothyroidism
SHBG	Sex hormone binding globulin
TH	Thyroid hormone
T4	Thyroxine
T3	Triiodothyronine
TBG	Thyroxine biding globulin
TRH	Thyroid releasing hormone
TSH	Thyroid stimulating hormone
TSH R	Thyroid stimulating hormone receptor
TH R	Thyroid hormone receptor
TPO-Ab	Thyroid peroxidase antibody
TRAb	Thyroid stimulating hormone receptor antibody

1. INTRODUCTION

THYROID

1.1.1 Thyroid hormones

Thyroid hormones (TH), thyroxine (T4) and 3,3',5-triiodo-L-tyronin (T3), are secreted from and stored in the thyroid gland. Thyroid hormones regulate energy, homeostasis, cell proliferation, and carbohydrate-, fat- and protein- metabolism.

1.1.2 The hypothalamic and the pituitary regulation of thyroid hormone secretion

The production of THs is mainly regulated by the hypothalamic – pituitary – thyroid axis [1]. Thyroid stimulating hormone (TSH) stimulates the production of TH in response to thyrotropin releasing hormone, produced by the hypothalamus. Thyrotropin releasing hormone (TRH) is transported to the pituitary via the hypothalamic hypophyseal portal system. TSH and TRH are regulated by negative feedback by T3 and T4. Furthermore, thyroid hormone levels are under influence of other hormones such as glucocorticoids, somatostatin, dopamine, prolactin, estrogen and growth hormones (Figure 1).

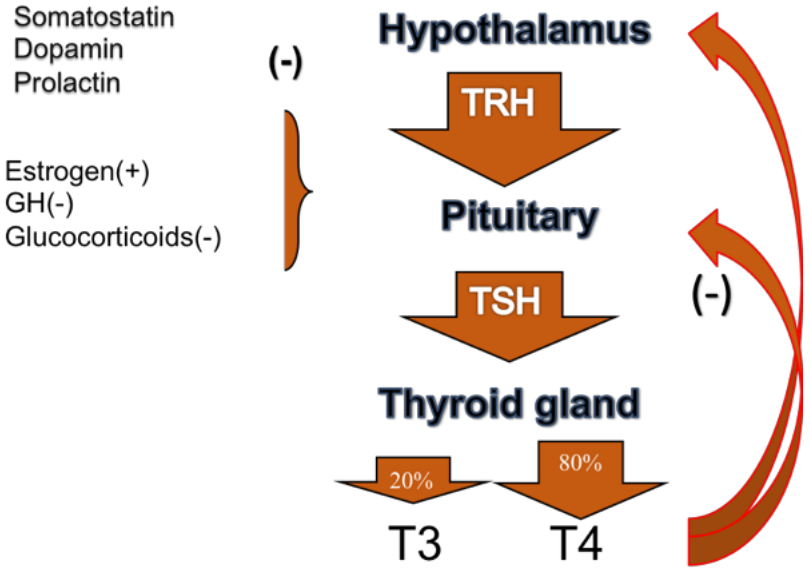

Figure 1. The figure shows the hypothalamic-pituitary-thyroid axis.

1.1.3 Thyroid stimulating hormone, TSH and TSH receptor

TSH is a heterodimeric glycoprotein hormone that shares the α-subunit with other glycoprotein hormones such as human chorionic gonadotrophin (hCG), follicle stimulating hormone (FSH) and luteinizing hormone (LH), but has a unique β-subunit. TSH exerts its effect by binding to the TSH-receptor (TSH R), which is located in the cell membrane of thyroid follicular cells. The TSH R is a member of the G-protein associated receptor family, similar to the hCG and LH receptors [2, 3]. TSH R expression has been shown in thyroidal tissue and also in extra-thyroidal tissues such as adipose tissue, testes, ovaries and endometrium [4, 5].

1.1.4 Thyroid hormone secretion

Follicular epithelial cells located in the thyroid gland produce thyroid hormones, mainly T4. They are hydrophobic hormones that are to more than 99 % bound to proteins, mainly to thyroxine binding globulin (TBG). The free fractions of thyroid hormones (fT4, fT3), which mediate thyroid hormone action in target cells, are estimated to 0.02 % of total T4 and 0.30 % of total T3.

The local enzymatic conversion of thyroid hormone in target tissues is regulated by iodothyronine deiodinases [6]. The majority of T3 in the circulation is derived from conversion of T4 by type 2 iodothyronine deiodinases (DIO2) and type 1 iodothyronine deiodinases (DIO1). The inactivation of T4 and T3 to reverse T3 (rT3) is mediated by type 3 iodothyronine deiodinase (DIO3) [7].

Cellular transport of thyroid hormone requires active transport across the plasma membrane. This is mediated through different members of mono carboxylate transporter (MCT) and organic anion transporting polypeptide (OATP) depending on target cells [8] .

1.1.5 Thyroid hormone receptor

Thyroid hormones exert their biologic effect through thyroid hormone receptors (TRs), which act as transcription factors to regulate gene expression [9]. TRs bind to a short DNA sequence of target gene called thyroid response element (TRE), which leads to transcription. By contrast, TRs interaction with the response elements, in absence of T3 leads to suppression of basal transcriptional activity [10] (Figure 2).

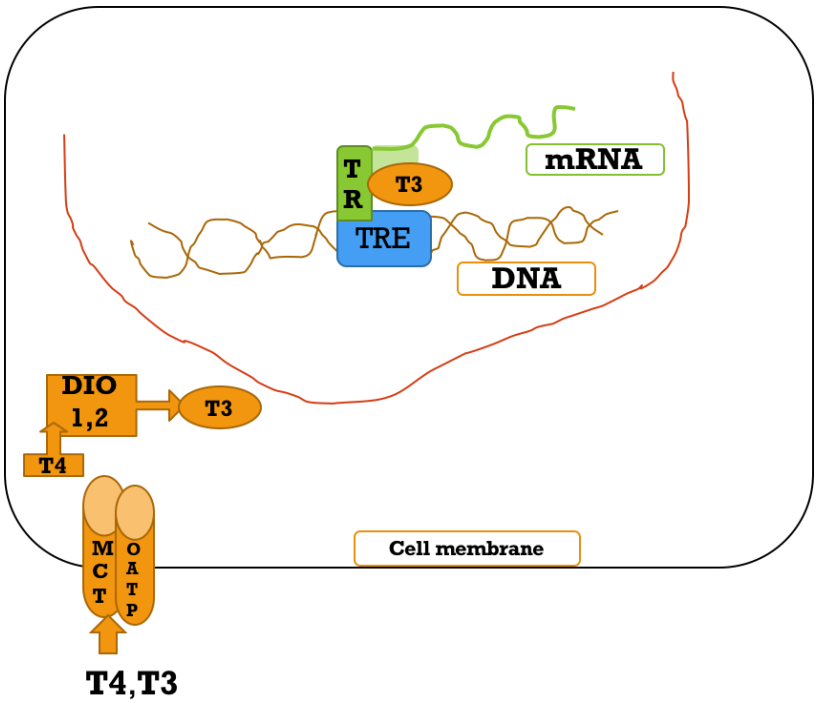

Figure 2. The diagram illustrates the action of thyroid hormone in the target cells. Thyroid hormone receptors (TRs), Thyroid response element (TRE), mono carboxylate transporter (MCT), organic anion transporting polypeptide (OATP), type 2 iodothyronine deiodinases (DIO2) and type 1 iodothyronine deiodinases (DIO1).

Thyroid receptors are encoded by two genes, TRα and TRβ, each with three isoforms, TRα1, TRα2 and TRα3 and TRβ1, TRβ2 and TRβ3 [11]. Thyroid receptors are expressed in most tissues and they have higher affinity to T3 than T4. TRα1 is predominantly expressed in brain, heart and skeletal muscles [11]. TRβ1 is widely expressed in different organs except testes[12] . TRα1, TRα2 and TRβ1 have been shown to be present in human endometrium [4, 5].

THYROID DYSFUNCTION

Change in serum concentration of TSH is the most commonly used indicator of thyroid dysfunction such as autoimmune thyroid dysfunction, hypothyroidism, subclinical hypothyroidism and hyperthyroidism.

1.1.6 Hypothyroidism

Hypothyroidism is defined as low levels of thyroid hormone combined with elevated levels of TSH. Hypothyroidism can be due to low secretion of the hormone from the thyroid gland, primary hypothyroidism, or due to low levels of TSH, that is central hypothyroidism. The worldwide prevalence of hypothyroidism is between 0.6 to 12 per 1000 women and 1.3 to 4 per 1000 men [13].

Iodine deficiency is the most common cause of hypothyroidism worldwide [14, 15]. In iodine sufficient countries like Sweden, the most common thyroid disorder is chronic autoimmune thyroiditis, usually known as Hashimoto's thyroiditis. The diagnosis of Hashimoto's thyroiditis is confirmed by the presence of anti-thyroid peroxidase antibodies (TPO-Ab) [16]. Hypothyroidism can also be caused by previous treatment of Graves´ disease such as anti-thyroid drugs, thyroidectomy or radioiodine treatment.

Symptoms of hypothyroidism are nonspecific and vary due to the severity of the disorder. Dry brittle hair and nails are common in these patients who may also have symptoms of chilliness, fatigue, weight gain and slowing of higher mental function. Treatment of hypothyroidism is thyroid hormone (L-T4) substitution.

1.1.7 Subclinical hypothyroidism

Subclinical hypothyroidism (SCH), defined as elevated serum levels of TSH combined with normal thyroid hormone levels [17]. Studies performed in the United States have shown a prevalence of 3 to 15 % of SCH. Women with SCH may have vague or nonspecific

symptoms or have symptoms similar to those with hypothyroidism. Women with TPO-Ab and elevated TSH levels are at higher risk of progressing from SCH to hypothyroidism and to development postpartum thyroiditis [18-21].

1.1.8 Hyperthyroidism (thyreotoxicosis)

Hyperthyroidism is defined as elevated thyroid hormone levels combined with almost undetectable levels of TSH. It affects approximately 2.0 % of women and 0.2 % of men worldwide. The most common type of hyperthyroidism is Graves´ disease. This condition is due to stimulation of thyroid gland by TRAb on the thyroid follicular cells [18].

Common symptoms of hyperthyroidism are weight loss, palpitations, tremulousness, heat intolerance, and anxiety. Physical findings such as tachycardia, thyroid enlargement and tremor are also seen. Treatment options are: anti-thyroid drugs, surgery and radioiodine treatment.

THYROID DYSFUNCTION AND FEMALE REPRODUCTION

1.1.9 Hypothyroidism

Women with hypothyroidism have low levels of sex hormone binding globulin (SHBG) and low levels of estrogen and testosterone [22]. Menstrual disturbances such as oligomenorrhea, amenorrhea and menorrhagia are common in hypothyroid women. These disturbances can partly be due to TRH-induced hyperprolactinemia and thus altered pulsatile GnRH secretion and partly due to defect hemostasis with low levels of coagulation factors. [23-25].

1.1.10 Hyperthyroidism (thyrotoxicosis)

Thyrotoxicosis may lead to different symptoms ranging from normal menstrual cycles to menstrual irregularities such as menorrhagia, oligomenorrhea, amenorrhea, anovulation and reduced fertility [26, 27]. Women with Graves´ Disease have 2 to 3 times higher serum levels of estrogen and LH during all phases of the menstrual cycle, probably due to high levels of SHBG [27]. The production of testosterone and androstenedione is also increased in these women [28].

THYROID AND PREGNANCY

1.1.11 The change in thyroid hormone production during pregnancy

During early pregnancy, a 2-fold estrogen derived increase in TBG occurs which requires an increase in thyroid hormone production and a higher daily intake of iodine [29-32] [33]. While the free fraction of thyroid hormone is slightly increased during the first trimester of pregnancy, the total serum levels of thyroid hormones are 1.5-fold higher in pregnant women than in non-pregnant women. The TSH levels have a transient fall during the first trimester of pregnancy due the thyrotrophic action of hCG [34], the lowest levels are seen around 10-12 weeks of gestation. In iodine sufficient areas the TSH levels will remain stable and similar to pre-gestational levels after the first trimester until the end of pregnancy (Figure 3).

1.1.12 The iodine status in pregnancy

A higher intake of iodine is required in pregnancy due to an increased distribution of iodine to the feto-placental unite and an increase in renal iodine clearance [44, 45]. According to WHO, a daily iodine intake of 250 µg is recommended for pregnant women, compared to a daily intake of 150 µg in non-pregnant women [46].

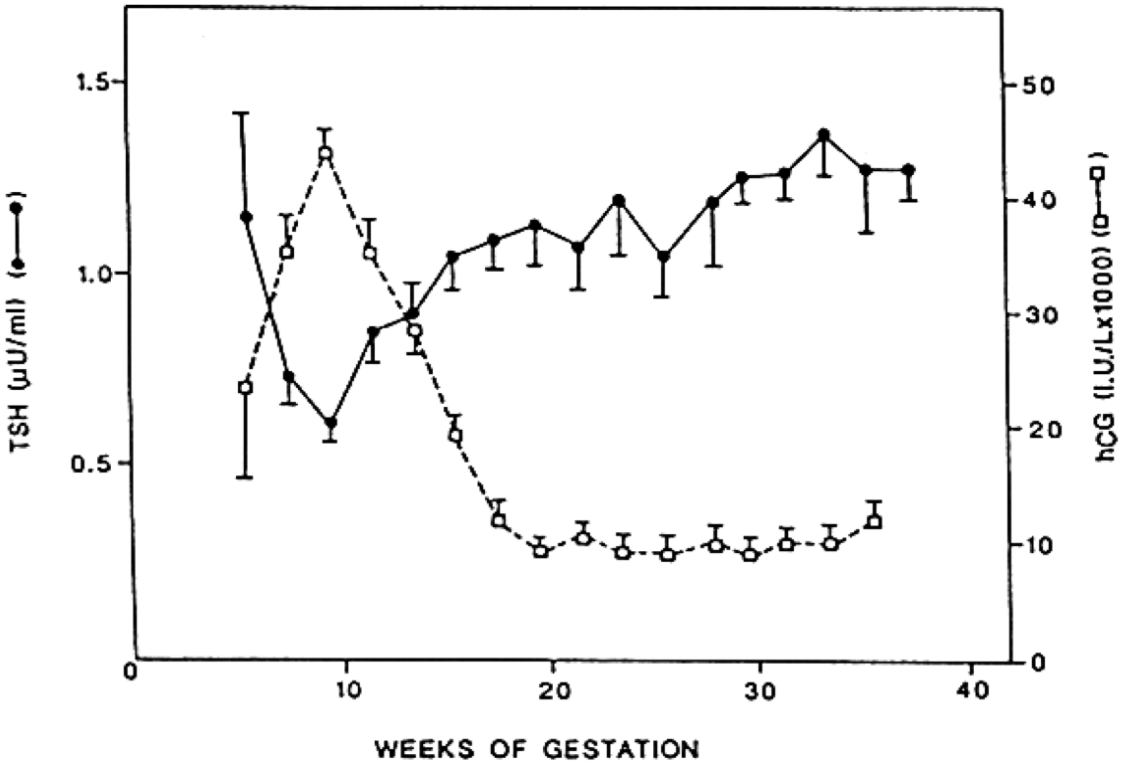

Figure 3. The diagram shows serum TSH and hCG as a function of gestational age, illustrated by Glinoer et al. JCEM 71:276 1990

1.1.13 Thyroid hormones and fetus

Despite incorporation of iodine late in the first trimester of pregnancy, the fetus does not start to secrete its own thyroid hormones until 18th to 20th weeks of pregnancy. Thus, the fetus is totally dependent on the trans-placental passage of maternal thyroid hormone during the early stages of pregnancy [35, 36]. During pregnancy, maternal thyroid receptor antibodies can also affect the fetus and anti-thyroid drugs due to trans-placental passage (Figure 3).

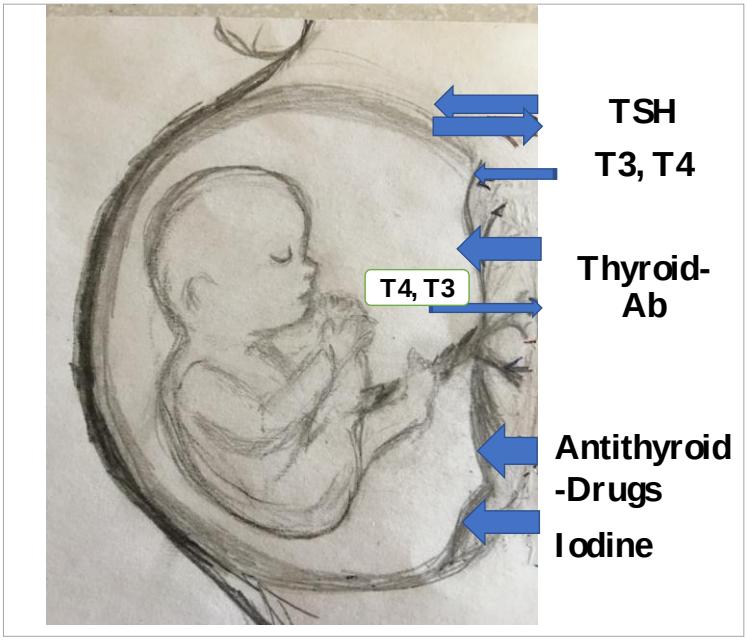

Figure 4. The figure shows trans-placenta passage of thyroid hormones and thyroid hormone related factors.

The fetus normal growth and neurologic development is dependent on optimal levels of thyroid hormones. Thyroid hormones influence neurodevelopmental events, such as neurogenesis, myelination, dendrite proliferation and synaptogenesis [37, 38].

THYROID DYSFUNCTION DURING PREGNANCY

1.1.14 Hypothyroidism / subclinical hypothyroidism or isolated hypothyroxinemia

Untreated hypothyroidism has been associated with an increased risk of obstetric and fetal complications while this it is not the case for isolated hypothyroxinemia or SCH [39, 40] [41]. Hypothyroidism and SCH have been associated with adverse fetal and obstetric outcome such as miscarriages, preterm labor before 32 weeks of gestation, postpartum

hemorrhage, respiratory fetal distress, intrauterine growth retardation (IUGR) and neurological disorders [39, 40, 42-46]. However, there is less evidence that untreated SCH during pregnancy is associated with neurological disorders in the fetus [47].

1.1.15 Hyperthyroidism

Untreated hyperthyroisim is associated with adverse pregnancy outcomes such as miscarriage, preeclampsia, IUGR and preterm delivery. Both thyroid antibodies and anti-thyroid drugs have the ability to pass through placenta. Thereby, women with positive TRAb have higher risk of fetal complications even in euthyroid status [48]. Pregnancy with positive TRAbs, require careful monitoring of thyroid status and TRAbs and controls of the fetus (ultrasonography) during pregnancy. The change of the immune system during pregnancy leads to remission in Graves´ disease like many other autoimmune diseases with a risk for a postpartum relapsing [49].

1.1.16 Gestational thyrotoxicosis

The production of TH increases during pregnancy due to secretion of hCG, with a peak during the 10^{th} to12^{th} weeks of gestation. hCG is a glycoprotein, which shares the alpha unites with TSH and can act as a TSH agonist. This leads to suppression of TSH and transient hyperthyroxinemia in the first trimester of normal pregnancy as well as multiple pregnancies and may cause hyperemesis gravidarum [50-52].

1.1.17 Postpartum thyroid dysfunction

Postpartum thyroiditis is a destructive autoimmune disease with a prevalence of 5 to 9 % and usually occurs within the first year after delivery. Women with diabetes mellitus type 1 have a threefold higher risk of developing postpartum thyroiditis. Women with positive

TPO antibodies have 50 % risk of developing postpartum thyroiditis and an increased risk of developing a permanent hypothyroidism [17, 53, 54].

2. FEMALE REPRODUCTIVE ORGANS

The female reproduction organs include vagina, uterus, fallopian tubes and ovaries, as illustrated in Figure 5.

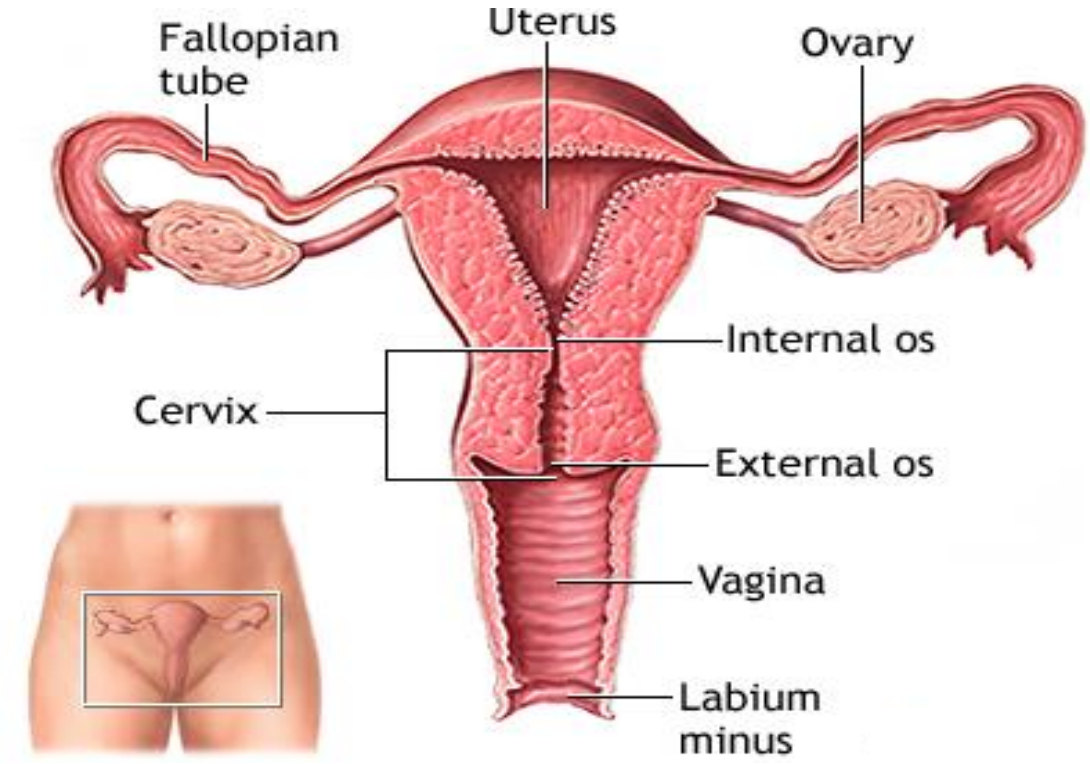

Figure 5. The female reproduction organs, (www.medlineplus.gov)

THE UTERUS

The uterus consists of three anatomical parts from cranial to the caudal: fundus, corpus uteri and cervix uteri. The upper left and right side of the uterus, from the uterine horns, continue into the Fallopian tubes. The uterine wall consists of three layers from inside out, a thin lining of cells called endometrium: a thick smooth muscular layer called myometrium and an outer thin serosal layer called serosa.

2.1.1 The Endometrium

The endometrium is a dynamic tissue that, in fertile women, undergoes cyclic regeneration, differentiation and shedding each month under the influence of the ovarian hormones [55]. The endometrium can be divided into two morphologic layers, the functional layer where the implantation of blastocyst takes place and the basal layer, from where the monthly regeneration of endometrium takes place. Histologically, the functional layer of the endometrium is divided into an epithelial surface, which constitutes of a luminal and glandular epithelium and a layer of stromal connective tissue. The luminal epithelium provides the initial site of implantation for the developing embryo. The epithelial layer secrets several factors including glycoproteins, cell adhesive molecules and glycogen during secretory phase which are required for endometrium development and implantation of the embryo [56]. The stroma is composed of fibroblasts, vessels and leukocytes that vary in their number and cell structure due to menstrual phase [57]. The fibroblasts and endothelial cells produced several factors involved in implantation of embryo including vascular endothelial growth factor (VEGF), cytokines and extracellular matrix components [58-60]. The endometrial leucocytes mediate pro-inflammatory conditions during secretory phase and are part of remodeling of the immune system associated to implantation, which includes, macrophages, neutrophils, mast cells, T, B cells and uterine natural killer cells.

THE FALLOPIAN TUBE

The Fallopian tubes are divided into four parts from the uterine horns to the ovaries: the interstitial part which transverses the uterine musculature, the isthmus and the ampulla which represents the major lateral part of the Fallopian tubes where fertilization of ovum takes place, and the infundibulum with its fimbriae where the released oocyte is captured (Figure 5). The Fallopian tube wall consists of three distinct layers; the serosa, the muscularis mucosae, which is composed of two layers: an outer longitudinal and an inner circular layer, and the mucosa. The mucosa consists of a luminal epithelial lining, which contains both secretory and ciliated cells. The fertilized oocyte transports throughout the fallopian tubes with help of beating motion of the cilia in combination with smooth muscular contractions [61].

THE OVARY

Each ovary is approximately four cm long and two cm wide. The outer layer of the ovary consists a single epithelium directly beneath that is a layer of connective tissue known as tunica albuginea. The ovarian stroma, consist of fibroblasts, collagen and elastin, forming the ovarian cortex. The ovarian medulla contains blood and lymphatic vessels.

The number of the follicles is highest prior to birth, approximately seven million, thereafter they are gradually reduced to about one million at birth, and around 24 000 at 37 years of age, where after the number of oocytes will decline even more rapidly [62]. At this age the number of follicles is normally so low that the possibility of pregnancy is significantly reduced.

2.1.2 Folliculogenesis

A cyclig recruitment of a pool of follicles, which will subsequently develop during folliculogenesis [63]. During the primordial follicle,premature follicle, will be develop into an antral follicle, which will either be selected to a dominant follicle or undergo atresia. Folliculogenesis occurs in the cortex of the ovarry and it takes almost one year for a primordial follicle to develop into an ovulatory follicle.The primordial follicle consists of a primary oocyte, a surrounding layer of granulosa cells and a basal lamina. The oocyte in the primordial follicle starts the first stage of meiosis, which arrests at the end of the diplotene of miosis until puberty. The first stage of meiosis will be completed before ovulation and the dominant follicle will start the second stage of meiosis, which will be completed after fertilization. During folliculogenesis, the granulosa cells proliferate due to influence of gonadotropins and ovarian hormones. The stromal-like cells, which surround the basal lamina, develop into two layers known as theca interna and theca externa [64].

2.1.3 The hypothalamus-pituitary regulation of sex steroid hormone secretion

The hypothalamus-pituitary-ovarian axis is a feedback system, which primarily regulates the menstrual cycle as it has been illustrated in figure 6 [65]. The two gonatrophins, follicle stimulating hormone (FSH) and luteinizining hormone (LH) regulate the secretion of ovarians hormones. The synthesis and secretion of these hormones by the anterior pituitary are regulated by a pulsatory secrition of hypothalamic-gonadotrophin releasing hormone (GnRH), which in turn is under influence of ovarians hormones. The main estrogen produced by the ovaries is estradiol, which have a direct feedback effect on the piutitary and the hypothalamos. During the late follicular phase, slithly after the peak of estrogen levels the peak of LH and FSH occurs which will induce ovulation. High levels of preogestrone postovulatory will suppress the gonadotropins (Figure 6) .

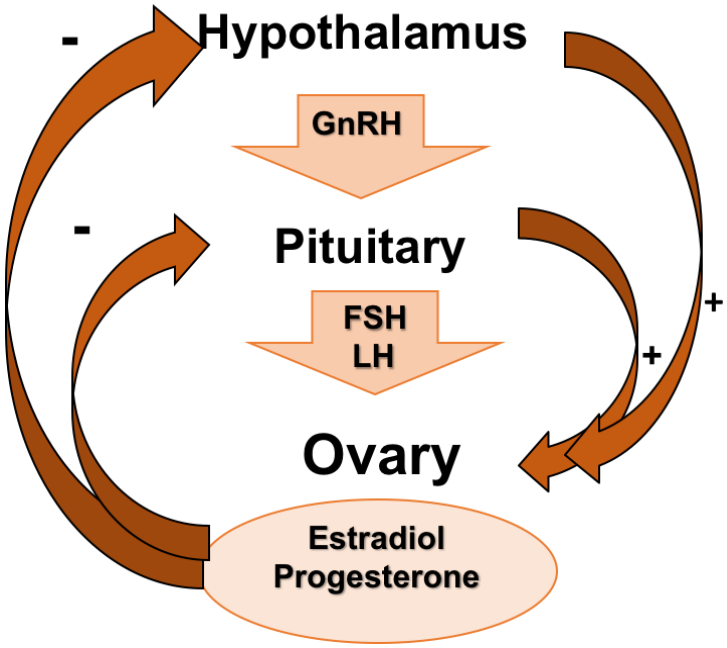

Figure 6. A diagram of the hypothalamus -pituitary -ovarian axis with positive (+) and inhibitory (-) feedback loop. Gonadotropins releasing hormone (GnRH), Follicle stimulating hormone (FSH), Luteinizing hormone (LH).

2.1.1 The ovarian cycle

The follicular phase, is initiated by increase in FSH during the preceding luteal phase, which stimulates folliculogenesis and secretion of the three estrogens, estrone, estradiol, estriol of which estradiol is the mainly produced. During this phase, the estradiol levels will gradually increase and reach its peak during the late follicular phase. The follicle with highest number of FSH receptors and the highest production of estrogens is selected as the dominant follicle [66]. The remaining follicles will undergo atresia. After the LH surge, the dominant follicle will mature and rupture, and thereby, the oocyte will be released [64, 67] [68] The residual follicle rearranges to form a corpus luteum which secrets both progesterone and estrogen (figure 7) [65]. In case of pregnancy, the secretion of progesterone continues from corpus luteum i.e., "corpus luteum graviditate", which resists 8 to 12 weeks when placenta take over the hormone production. In absence of a pregnancy, the corpus luteum vill degenerate and form a corpus albicans. The drop of progesterone and estrogen levels eleminate the negative feedback to the hypothalamo-pituitary axes (figure 6 and 7).

Besides estrogen and progesterone, the ovary produces testosterone and other steroid and non-steroidal hormones such as inhibin A, B and anti-Müllerian hormone [69]. The anti-Müllerian hormone is produced by the granulosa cells of the primordial and antral follicles, with a stable serum concentration during the menstrual cycle [70].

2.1.2 Endometrial differentiation during the menstrual cycle

The first menstruation, menarche, starts at the age of 8.5 to 13 years, at mid to late puberty. The ideal cycle length is 28 days, while the menstrual cycles in fertile women range from 21 to 34 days generally due to the variability during the follicular phase. During the menstrual cycle the monthly preparation of the endometrium for implantation occurs.

The menstrual cycle can be divided into three phases due to endometrial changes: one degenerative phase called menstrual phase, one proliferative phase which corresponds to the ovarian follicular phase, and one secretory phase, which corresponds to the ovarian luteal phase [71]. The *menstrual phase* of the menstrual cycle is initiated by bleeding, due to contraction of blood vessels, which will results in necrotic changes and degeneration of the functional layer of the endometrium. The regeneration of endometrium starts with the gradual

rise in estrogen during the *proliferative phase*, which initiates mitotic activity of all cell components of the endometrium, which leads to a considerable thickening of the endometrial mucosa. After ovulation, the *secretory phase*, the production of both progesterone and estrogens by corpus luteum increase. During this phase the endometrium will be prepared for implantation of the embryo. Decidualization of the stromal cells which include edema, coiling of spiral arterioles, and remodeling of the stromal cells to secretory epithelioid-like decidual cells [72]. As a part of decidualization, a remodeling of maternal blood vessels occurs, which initiates infiltration and / or proliferation immune cells, primarily macrophages and uterine natural killer cells [73, 74]. These immune cells secrete metalloproteinase and cytokines that degrade the extracellular matrix [59, 75]. Progesterone is essential for maintenance of the decidualization of the estrogen-primed endometrium. In absence of pregnancy, degeneration of the endometrium starts after accumulation of inflammatory cells in the endometrium. During the late secretory phase, degeneration of the endometrium starts, the secretion of estrogen and progesterone has dropped to low levels, and the endometrium begins to shed [76, 77](Figure 7).

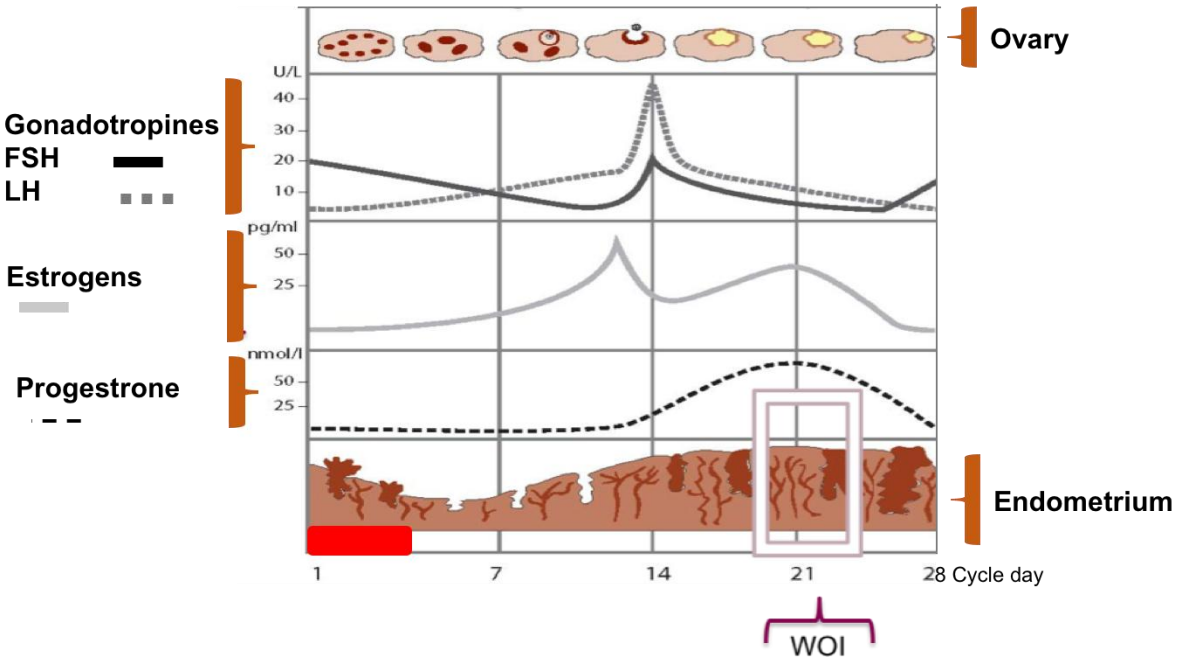

Figure 7. The menstruation cycle and its related hormones: Gonadotropic hormones; Follicle stimulating hormone (FSH), Luteinizing hormone (LH). Gonadal hormones (estrogen and progesterone), Implantations window (WOI).

3. IMPLANTATION

Women have an estimated implantations rate of less than 30 % in a natural menstrual cycle. Implantation requires fertilization of a haploid oocyte by a haploid sperm in the Fallopian tube, development of the early embryo during its transport in the Fallopian tube and finally implantation of the embryo into the endometrium [78, 79].

The fertilization of oocytes is initiated by fusion of membranes of sperm and oocyte when the sperm enter the oocyte forming zygote. This event takes place in the ampulla of the Fallopian tube. The embryo undergoes regulated mitotic cleavage and different developmental stages (morula, blastocyst) during its transport through the Fallopian tube (Figure 8).

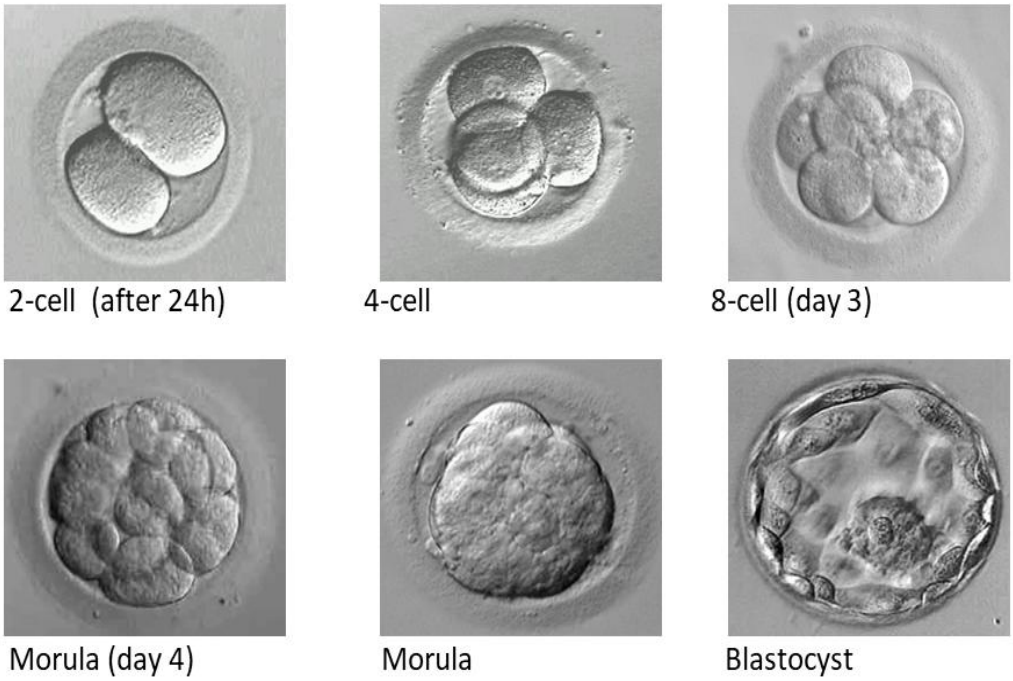

Figure 8. The figure shows different developmental stages of human embryo.

The blastocyst consists of an outer trophectoderm, an inner cell mass and a fluid-filled cavity i.e., the blastocyst cavity. The inner cell mass will develop into an embryo and its belonging structures like the amnion and the yolk sac while the trophectoderm will form the placenta.

The endometrium is only receptive for the embryo during a limited time of the mid secretory phase i.e., “the implantation window” (WOI). During this phase, assumingly 7 to 9 days after the LH surge, the endometrium is transformed to a receptive phase under influence of progesterone. Implantation beyond this transient receptive period has been associated with

early pregnancy loss. A morphologic change in the endometrium during WOI is the appearance of pinopodes, which are cytoplasmic protrusions on the surface of endometrium, which have been proposed to be structural markers of endometrial receptivity. Many potential biomarkers that belong to different functional groups have been identified to be involved in implantation [80]. However, no specific biomarker for human implantation has yet been identified.

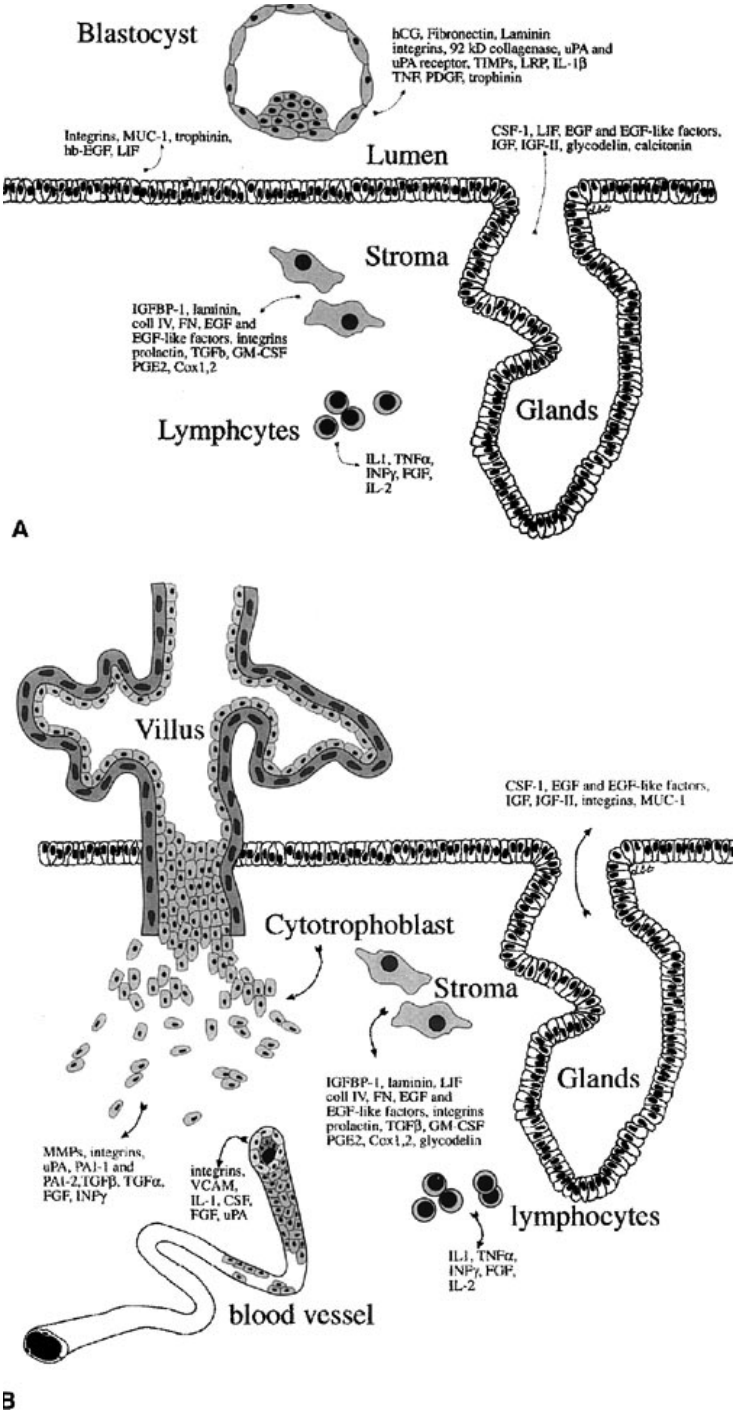

Figure 9. The figure shows the embryo-endometrial dialogue (A) before apposition and (B) after invasion. Groll J, Lessey B, *Glob. Libr. women's med., (ISSN: 1756-2228) 2009; DOI10.3843/GLOWN.10318*

Around day 4 to 5 after fertilization, the morula enters the uterine cavity (Figure 8). Thereafter, the developing blastocyst orients towards the surface of the endometrium and forms a loose connection with the endometrium, named apposition [81]. Adhesion is the result of a stronger attachment of the embryo into the endometrium by penetration of trophoblast cells into the luminal epithelium. Thereafter the trophoblast cells will differentiate into cytotrophoblasts at the inside and syncytiotrophoblasts at the outside. The extravillous trophoblasts will breach into the uterine blood vessel walls to ensure provision of placenta blood supply. Soon thereafter syncytiotrophoblast cells will penetrate deeper in the endometrium beneath the decidua cells [82, 83]. Normal development of these events requires a dialog between the blastocysts and the decidualized endometrium the so called "cross-talk" (Figure 9) [84-86]. This dialog is mediated by several different molecules, including hCG interleukins and leukemia inhibitory factor (LIF), secreted both in paracrine and autocrine manners from the blastocyst and the endometrium [58, 81, 87-90].

MEDIATORS OF IMPLANTATION

Many potential biomarkers belong to different substances such as growth factors, adhesions molecules and prostaglandins, which have been proposed to be involved in implantation. Since the study of human implantation is unethical and therefore impossible, most information has been derived from cell culture and animal studies. One approach to reach more knowledge about implantation is by using gene expression arrays and bioinformatic tools to identify the transcriptomic signature of the receptive endometrium [91]. Altmäe and collogues have identified a network of molecules involved in implantation, which includes cytokines and their interactions with the cytokine receptor [92]. Chemokine receptors are not only expressed in leucocytes but also in human blastocyst and endometrium both prior to implantation and during implantation. These chemotactic proteins are involved in several important processes of implantation such as, angiogenesis, embryo development and maintenance of innate and adaptive immunity [86, 93]. One inflammatory mediator of implantation is IL8. The IL8 is involved in processes important for implantation such as chemotaxis, neutrophil activation and angiogenesis. Its' receptors, CXCR1, has been seen to be up regulated in presence of a blastocyst [94]. Chemokines are important modulators for migration and activation of leukocytes particularly uNK cells and macrophages. The uNK cells and macrophages are involved in adaptation of immunity during pregnancy i.e.,

fetomaternal tolerance, which is essential for a successful implantation of the allogeneic fetus and placenta [95, 96]. One of the known mechanisms in this process is an epigenetic change in stromal decidual cells, which leads to modulation of their capacity to produce chemokines responsible for T cell recruitment [97].

3.1.1 Genetics factors associated with infertility

Genetic variations have been proposed to be involved in infertility and recurrent miscarriage [98-100]. The genomic material in all living organisms is made from four nucleotides: adenosine, cytidine, guanosine and thymidine. The inter-individual differences consist of a few variations in the DNA sequences in the human population [101]. When a single nucleotide in a DNA sequences is altered called SNP (single nucleotide polymorphism). However, there are only a few SNPs, which have impact on cell function and human health.

A polymorphism in regulatory region of *HABP2* gene, genotype in rs2240879 and rs1157916, has been associated to infertility [102]. The HABP2 gene contains 13 exons and spans 35 kb in chromosome region 10q25 to q26. One known polymorphism in the *HABP2* gene is Marburg I, SNP rs7080536 (Gly534Glu), in axon 13. The Marburg I, SNP has a frequency of 4.3 to 9% in the Caucasian population which is a result of replacement of a single nucleotide G by A in the protease domain. The Marburg I, SNP is associated with low proteolytic activity of the HABP 2 and thereby increases risk for late complications of carotid stenosis [103, 104].

The *HABP2* gene is one of the genes that have been identified to be involved in endometrial receptivity and female infertility. The *HABP2* gene encodes HABP2 protein, also known as activators of coagulation factor VII, urinary plasminogen activator (UPA) and tissue factor pathway inhibitor (TFPI) [105, 106]. The HABP2 protein is involved in coagulation, fibrinolysis, tissue remodeling and acts as a vascular inflammatory factor. Furthermore, it has also been shown to be negatively involved in vascular integrity. In the female reproductive system, HABP2 protein is an extracellular serine protease, which binds hyaluronic acid and functions as an angiogenesis promoter in the extracellular matrix with a cyclic variation during the menstrual cycle [107]. The highest levels of HABP2 are correlated to time of growth and remodeling during the mid-proliferative and mid-secretory phase of the menstrual cycle [108]. A lower level of HABP2 in the endometrium in women with unexplained

infertility has been observed. Lower endometrial gene expression of HABP2 has even been observed in women with recurrent miscarriage [107, 109].

4. INFERTILITY

Infertility has been defined as the inability to conceive after one year or more of regular intercourses without use of contraception. The monthly fecundity rate in fertile couples has been estimated to 10 to 15 % [110, 111]. Between 2 to 10 % of couples worldwide are unable to conceive a child while more than 10 to 25% suffer from secondary infertility, i.e. inability to conceive after one or more successful pregnancies. Infertility has strong association with mental disorders like depression and anxiety and psychosocial distress [112]. The American Society for Reproductive Medicine (ASRM) and the World Health organization (WHO) consider infertility as a reproductive disease and has recommended investigation and treatment after one year for women below the age of 35 years and six months for women over the age of 35 years.

Infertility can be related to male or female factors or combinations of both. The female factors include: endocrine dysfunctions such as ovarian dysfunction including luteal phase deficiency, prolactinoma, thyroid dysfunctions and anatomical abnormalities such as tubal and peritoneal factors due to post inflammatory conditions. Other factors associated with infertility in women are endometriosis, polycystic ovary syndrome and thrombotic inherited diseases.

The male factors include anatomical abnormality, previous infections, sperm abnormality, chromosomal abnormalities and lifestyle such as cigarette smoking and alcohol consumption [113]. The clinical examination and tests that couples undergo to detect possible etiology for infertility include semen analyses, with normal results according to the World Health Organization (WHO) criteria [114, 115]. Women need to have normal levels of serum TSH, prolactin, normal ovulation and a normal tubal passage either diagnosed by hysterosonosalpingography or laparoscopy on suspicion of endometriosis [116].

Assisted reproductive technology (ART) involves methods used for treatment of couples with infertility such as clomiphene citrate or gonadotropin treatment in combination with intrauterine insemination, in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Since development of IVF in 1978, which was acknowledged by awarding Dr. R. G Edwards the Nobel Prize in the 2010, the number of ART children has increased. Despite continuous development and optimization of ART, not all attempts to treat are successful [117, 118].

UNEXPLAINED INFERTILITY

In more than 10 to 30 % of cases the etiology of infertility remains unexplained [119, 120] and defines as unexplained infertility. An individualized management of couple suffering from unexplained infertility has been recommended with opportunity to offering current treatment options including, expectant management, changing lifestyle and ART.

RECURRENT MISCARRIAGE

One of the most common early pregnancy complications is loss of pregnancy during the first 20 weeks of pregnancy i.e. miscarriage, which occurs in approximately 15 % of all clinically confirmed pregnancies. Recurrent miscarriage is less common and affects 1 to 3 % of couples trying to become pregnant. It has been defined as three or more consecutive pregnancy losses up to the 20th week of pregnancy (www.eshre.eu).

Recurrent miscarriage has been related to multiple factors that factors include endocrine disorders such as untreated hypothyroidism, uncontrolled diabetes mellitus, luteal phase defect, polycystic ovarian syndrome (PCOS) and hyperprolactinemia. Other factors associated with recurrent miscarriage are anatomic abnormalities. Furthermore, inherited diseases such as autoimmunity including thyroid autoimmunity, antiphospholipid antibody syndrome (APS) and parental chromosomal abnormalities, antithrombotic inherited diseases such as factor V Leiden, mutation in the promoter region of the prothrombin gene and mutations in the gene encoding methylene tetrahydrofolate reductase (MTHFR) resulting to hyperhomocysteinemia, protein S and protein C and deficiencies in antithrombin, has been related to recurrent miscarriage. Although, none of these factors has been proven to cause recurrent miscarriage, they have only been suggested to increase the risk for recurrent miscarriage [121]. In majority of cases, approximately, in 50 % of cases the etiology of pregnancy losses remains unclear, which defines as unexplained recurrent miscarriage [122, 123].

5. AIM OF THE STUDIES

GENERAL AIM

The overall aim of the studies was to explore endocrine and paracrine factors related to female reproduction.

SPECIFIC AIMS

-To detect the incidence of subclinical hypothyroidism and hypothyroidism in women during early pregnancy.

- To detect associations between genetic variations in *HABP2* gene with recurrent pregnancy loss.

-To compare the endometrial gene expression profile in women with unexplained recurrent pregnancy loss with fertile controls.

- To compare serum and endometrial levels of thyroid related factors in fertile women and women with unexplained infertility.

- To explore the presence and distribution of proteins related to thyroid function in endometrium and Fallopian tubes

- To study the influence of T4 supplementation on in vitro development of the embryo.

6. MATERIALS AND METHODS

ETHICS

Study II and I were approved by the Regional Ethical Review Board in Stockholm (2009/2:6) and (2009/177-32) respectively. Study III was approved by the Regional Ethics Board in Stockholm and the Regional Ethical Board in Uppsala (2006/751-31/1-4 and 2009/498-32). All participants gave informed oral and written consent prior to participation. Study IV was approved by the Regional Ethical Board in Stockholm and the Regional Ethical Board in Uppsala (2008/046/1 and 2012/339). The women who donated Fallopian tubes and endometrium gave their informed consent before participation. The human embryos were donated for research after 5 years limit storage has passed. Both partners of the couples involved signed an informed consent form after receiving oral and written information. All research was performed in accordance with relevant guidelines/regulation.

MATERIAL

6.1.1 Incidence of subclinical hypothyroidism and hypothyroidism during first trimester of pregnancy

Women (n = 1298) up to 12 weeks and 6 days of pregnancy were screened for thyroid dysfunction except for hyopthyroxineamia. The women were enrolled at three participating antenatal care unites, two in the north of Stockholm, in high- income areas, and one in the south of Stockholm, at a mixed socioeconomic area. The incidence of thyroid dysfunction in a high risk group (n = 88), only women at risk of thyroid disease, was compared to a low risk (n = 511) and a general screening group (n = 699). The criteria for high-risk group were based on recommendation for screening, in Stockholm, at the time of the study, including, family history of thyroid dysfunction or previous treatment with LT4, propylthiouracil, methimazole, radioiodine, previous surgery, goiter or clinical suspicion of thyroid disease. Women with diabetes severe cardiovascular diseases and Systemic Lupus Erythematosus were not included in this study as they were usually attending specialist care units (Table 1).

6.1.2 HABP2 polymorphisms in women with recurrent miscarriage

Women with recurrent miscarriage

Women (n = 165) with a diagnosis of recurrent miscarriage, during 1989 to 2009 were identified from outpatient registers, from the departments of Obstetrics and Gynecology at Uppsala University Hospital, Karolinska University Hospital (Huddinge and Solna), and Danderyd University Hospital. A careful review of medical journal of women with recurrent miscarriage was performed. All women with recurrent miscarriages had at least three or more consecutive miscarriages in the first or second trimester of pregnancy. Women with known risk factors for recurrent miscarriages such as, type I diabetes or other endocrine dysfunctions, Systemic Lupus Erythematosus, severe thrombophilia, and major chromosomal aberrations (in either partner) were excluded. Additionally, all women included in the study answered a questionnaire on reproductive health.

Control women

The control group (n = 289) were matched for age at their first pregnancy and consisted of heathy fertile women without previous history of miscarriage with at least one successful pregnancy. All women included in this study had conceived naturally and were of Caucasian ethnicity or European origin.

6.1.3 Endometrial gene expression in women with recurrent miscarriage

Women with recurrent miscarriage

Women with recurrent miscarriage (n = 10) were recruited at departments of Obstetrics and Gynecology at Danderyd University Hospital from 2008-2013. The women with three or more verified consecutive miscarriages in the first or second trimester of pregnancy were included. Cases with obvious causes for recurrent miscarriages including chromosomal anomalies in the couple, uterine structural abnormalities, thrombophilic disorders and the presence of anti-phospholipid antibodies in serum were excluded. Additionally, women with disease linked to recurrent such as obesity, poorly controlled diabetes, thyroid disease or pelvic infections were excluded.

Control women

Endometrial biopsies were obtained from healthy fertile women (n = 7) volunteers with regular menstrual cycles between 28 and 30 days. All samples were collected at the

department of Obstetrics and Gynecology at Danderyd University Hospital. These women were either undergoing laparoscopic sterilization or were recruited among the personnel in the hospital. All controls were fertile with at least one successful pregnancy and no history of recurrent miscarriage. All samples in both groups were collected at five to eight days after the LH surge (Table 1).

6.1.4 Thyroid hormone in infertile women compared to fertile controls and thyroid associated protein in endometrium Fallopian tube and embryo

6.1.4.1 TSH, fT4, and TPO-Ab serum levels in women with unexplained infertility compared to controls

Women with unexplained infertility

Serum levels of fT4, TSH and TPO-Ab were compared in women with unexplained infertility (n=147) and women with proven fertility (n = 67). All samples were collected at Kvinnohälsan, Karolinska University Hospital, Huddinge, at Danderyd University Hospital and at the Center of Reproduction at Uppsala University Hospital. All women included in this study were healthy and had a normal menstruation cycle between 28 to 32 days (Table 1). The couple with unexplained infertility had complete normal examinations with at least two normal semen analyses (based on WHO 2010 criteria). The women were normo-ovulatory had normal hormone analyses and normal tubal patency and no signs of endometriosis.

Control women

The control group was healthy volunteers with at least one child born after spontaneous conception

6.1.4.2 Endometrial biopsies from infertile and fertile women for detection of thyroid related proteins

Women with unexplained infertility

Endometrial biopsies from women with unexplained infertility (n = 19) were compared to endometrial biopsies from healthy fertile controls (n = 28) Samples from women with infertility were obtained at Kvinnohälsan, Karolinska University Hospital, Huddinge. All samples included in this study were collected during receptive phase (LH+ 6 to LH+ 8) (Table.1).

Control women

The women in control group (n = 28) were healthy with at least one child born after spontaneous conception. These women were enrolled at the Department of Obstetrics and Gynecology, Karolinska University Hospital, Huddinge.

6.1.4.3 Fallopian tube in fertile women

Fallopian tubes were collected from healthy volunteers (n = 13) with proven fertility, for detection of thyroid related protein during menstrual cycle. These women were attending Uppsala University Hospital for laparoscopic tubal sterilization or hysterectomy due to leiomyoma (Table 1).

Table 1. Characterization of study participants included in the study. Data is presented as median and range. Statistics according to Chi-square, Mann-Whitney rank sum test and Kruskal Wallis test.

TSH during first trimester				
Serum samples	**Low risk** (n = 511)	**High risk** (n = 88)	**General** (n = 699)	**p-value**
Age	33 (17 – 40)	35 (22 – 44)	30 (16 – 50)	0.001
BMI	23 (17 – 46)	23 (15 – 34)	-	0.718
Polymorphism in *HABP2* gene				
Blood samples	**Fertile** (n = 289)	**Recurrent miscarriage** (n = 165)		**p-value**
Age	30.1 ± 5.8	30.3 ± 5.9		0.979
Number of children	1.5 ±1.2	2.3 ± 0.96		<0.001
Gene expression array				
Endometrial biopsies	**Fertile** (n = 7)	**Recurrent miscarriage** (n = 10)		**p-value**
Age	37 (28 – 42)	36 (28 – 40)		0.417
fT4, TSH, TPO-Ab				
Serum samples	**Fertile** (n = 67)	**Infertile** (n = 147)		**p-value**
Age	36 (24 – 44)	34 (22 – 40)		0.899
BMI	23 (18 – 33)	23 (17 – 37)		0.512
TRα1, TRβ1, TSH R, DIO2, MCT8				
Endometrial biopsies	**Fertile** (n = 28)	**Infertile** (n = 19)		**p-value**
Age	36 (25 – 41)	35 (31 – 39)		0.208
BMI	25 (23 – 27)	22 (18 – 29)		0.475
TRα1, TRβ1, TSH R, DIO2				
Fallopian tube	**Follicular phase** (n = 6)	**Luteal phase** (n = 7)		**p-value**
Age	40 (37 – 44)	41 (39 – 46)		0.295
BMI	29 (23 – 37)	28 (21 – 36)		0.413

6.1.5 Human embryos

The blastocyst stage human embryos (n = 74) were donated for research by couples undergoing in vitro fertilization treatment at Uppsala University Hospital. The embryos were suitable for transfer on thawing and graded by an experienced embryologist

6.1.6 TSH screening in early pregnancy

Blood samples from pregnant women were collected at the first visit at each antenatal care units. TSH levels were analyzed in serum according to clinical routine at Karolinska University Hospital in either Solna (Beckman Coulter AB, a chemiluminescent immunoenzymatic one-step sandwich assay) or Huddinge (immunoassay, Modular E 170; Roche). Subclinical hypothyroidism was defined according to locale clinical guideline at the time of study.

6.1.7 Analysis of polymorphism in HABP2 in women with recurrent miscarriage

Peripheral blood samples of women with recurrent miscarriage and controls were collected in EDTA tubes at each center and stored at − 20 °C until all samples were collected. After extraction of genomic DNA from whole blood in accordance to the QIAamp® DNA Blood Midi/Maxi handbook (QIAGEN, Netherlands), three polymorphisms in *HABP2* gene; rs1157916 in the promoter area, rs2240879 in 5'UTR and rs7080536 (Gly534Glu) in exon 13 were analyzed. Real-time PCR was carried out by use of TaqMan® SNP Genotyping Assays according to the manufactures instructions (Applied Biosystems, USA). The SNPs were chosen based on data from literature and from NCBI database. PCR was performed on Step One PlusTM Real-Time PCR System (Applied Biosystems, Foster City, USA).

6.1.8 Endometrial gene expression in women with recurrent miscarriage

Collection of endometrial samples

All endometrial biopsies were obtained during mid secretory phase. For determination of ovulation a self-testing luteinizing hormone (LH) test of the morning urine (Clearplan; Unipath Ltd., Bedford, United Kingdom). The ovulation was furthermore conformed by visualization of a corpus luteum by ultrasound and if it necessarily, by including measurement of elevated serum progesterone concentrations (>25 nmol/L). The biopsies were obtained using pipelle aspiration (Pipelle de Cornier, Laboratorie C.C.D., Paris, France).

The biopsies were then immediately placed in RNAlater (Qiagen, Hilden, Germany) and stored at 8°C for 24 h and thereafter at −20°C until further processing. All biopsies were used for gene expression by gene expression array.

Gene expression analyses

Total RNA was isolated by use of miRNeasy, (Qiagen, Hilden, Germany) and 250 ng was used to generate amplified and biotinylated sense-strand cDNA. The arrays were then scanned using the GeneChip® Scanner 3000 7G Affymetrix. Raw data from the microarray analysis were normalized and the expressed genes were subsequently analyzed by the statistical computing language R (www.r-project.org) using packages available from the Bioconductor project (www.bioconductor.org). An empirical Bayes moderated paired t-test was used, the differently expressed genes between two groups were detected (p-value <0.05). The differently expressed genes by an average of log2 fold change of more than ±1 was selected. Cluster and principal component analysis were performed for visualization of differentially expressed genes by using genesis version 1.7.6 [124]. The functional analysis and gene ontology were studied by use of Database for Annotation, Visualization, and Integrated Discovery (DAVID) with the whole genome as background. The networks of the differentially expressed genes were identified by used of Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com).

6.1.9 Thyroid hormone levels and thyroid related proteins in women with unexplained infertility compared to healthy controls

TSH, f T4, TPO-Ab in serum

Blood samples (serum) in fertile and infertile women were collected and stored at -20°C until analysis. All samples were analyzed by use of Enzyme linked immunosorbent assay (ELISA) according to instruction from manufacturer for fT4, TSH and TPO-Ab.

Collection of endometrial samples

All women included in this study were monitored by vaginal ultrasonography for evaluation of ovulation, by measuring the endometrial thickness and the leading follicle from

cycle day 10 until development of a corpus luteum; serum progesterone was measured in the mid-luteal phase. For determination of the day of the LH surge, a self-reporting luteinizing hormone (LH) test of the morning urine (Clearplan; Unipath Ltd., Bedford, United Kingdom) was used. Endometrial biopsies were obtained using Pipelle device as per standard protocols. All endometrial samples were immediately fixed in 4 % formaldehyde for a maximum 24 hours and then stored in 70 % ethanol until embedding in paraffin. Endometrial biopsies were analyzed using immunostaining. The presence and distribution of TSHR, TRα, TRβ, DIO2 and MCT8 was detected.

Fallopian tube samples

Fallopian tube samples were used for detection of TRα1, TRβ1, TSH R and DIO2 in different parts and compartments. These samples were obtained either during laparoscopic tubal sterilization or hysterectomy due to leiomyoma. Six samples were taken during the follicular phase (cycle day 1-13) and seven samples were during the luteal phase (cycle day 14-28). The tissue samples were fixed in 4 % formaldehyde and then stored in 70 % ethanol until embedding in paraffin. Immunohistochemistry was used for detection of TSHR, TRα, TRβ and DIO2.

Immunohistochemistry

Immunohistochemistry was used for detection and location of thyroid related proteins in tissue from endometrium and Fallopian tube. In brief, a specific primary antibody interacts with an antigen in the tissue (Table 2). For detection a biotinylated secondary antibody conjugated and thereafter visualized by enzymatic activation of a chromogenic substrate was used.

6.1.9.1 Embryo Culture

Frozen-thawed embryos were used. After thawing, the embryos were randomly allocated to be cultured in standard media with T4 added to a final concentration of 20 pmol/L (n=38) or in standard culture media (n= 36) until day 6 and thereafter blastocyst formation was determined according to Gardener, based on blastocyst development, quality of inner cell mass and quality of trophectoderm.

Table 2. Antibodies used in immunohistochemistry.

Antibody	Receptor	Concentration
Polyclonal Rabbit IgG	TRα1	5 µg/ml
Monoclonal mouse IgG	TRα2	5 µg/ml
Polyclonal Rabbit IgG	TRβ1	3 µg/ml
Monoclonal mouse IgG	TSHR	2.5 µg/ml
Polyclonal Rabbit IgG	DIO2	50 µg/ml
Polyclonal Rabbit IgG	MCT8	50 µg/ml

For evaluation of immunohistochemistry, two observers evaluated the staining intensity of each sample. Staining intensity and the number of stained cells were graded according to the following: 0 = no staining, + = faint staining, ++ = moderate staining, and +++ = strong staining.

6.1.10 Statistical Analysis

For descriptive statistics, independent t-test or Mann-Whitney Rank Sum Test were used. For comparisons between three or more groups, chi-square test was used in the study I, II, III and Kruskal-Wallis test followed by Dunn's test was used in the study IV. For differences within or between groups in study II one-way ANOVA Test was used and for allele frequencies deviations from Hardy-Weinberg equilibrium were investigated. In study III non-parametric correlations were tested according to Spearman's rank correlation. For the number of median and good quality embryos, Fischer's exact test was used. All differences with p values ≤ 0.05 were considered statistically significant.

7. RESULTS

7.1.1 Incidence of hypothyroidism and subclinical hypothyroidism in early pregnancy

The incidence of subclinical hypothyroidism and hypothyroidism was almost the same, (almost 10), in all three groups regardless of TSH levels ≥ 2.0 mIU/L or ≥ 2.5 mIU/L in early pregnancy (Figure 10).

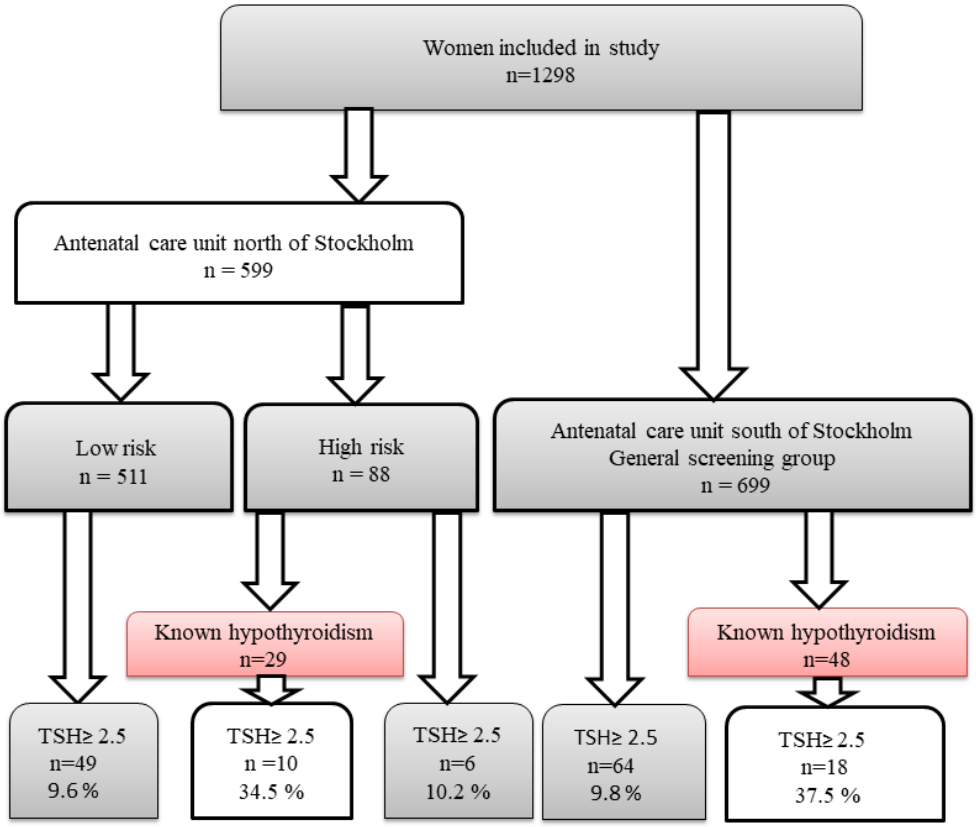

Figure 10. Flow diagram of study population of early pregnant women and the incidences of subclinical hypothyroidism and hypothyroidism

Hypothyroid women on LT4 treatment prior to pregnancy (n=77) had in 50.6 % of cases a TSH level ≥ 2.0 mIU/L and in 36.4 % of cases a TSH levels ≥ 2.5 mIU/L.

During this study, all women with subclinical hypothyroidism were treated with LT4 and monitored with control of fT4, TSH during pregnancy with a goal of treatment at TSH level < 2.0 mIU/L.

7.1.2 Polymorphism in *HABP2* genes in women with recurrent miscarriage

In the present study, there were no significant differences in the three studies polymorphisms of the *HABP2* gene observed between women with recurrent miscarriage and fertile controls. However, the rs1157916A allele showed a tendency to be more frequent among women with recurrent miscarriage (P = 0.058).

The majority of women with recurrent miscarriage (77 %) eventually had a full-term pregnancy with at least one child successfully born. Women with five or more miscarriages had less chance, 26.2%, of a successful pregnancy. The live birth rate in study population is shown in table 3.

Table 3. Table shows live birth in study population, unexplained recurrent miscarriage (RM) and control. Data is presented as mean ± SD, RM divided in three groups due to age

	Recurrent miscarriage				**Control**
Age at first miscarriage	21.2 ± 2.3	29.8 ± 2.7	37.3 ± 1.8	All	
No. of patients n, (%)	27 (16.4 %)	95 (57.6 %)	43 (26.0 %)	165	289
Children before miscarriage	4 (14.8 %)	31 (32.6 %)	17 (39.5 %)	52 (31.5 %)	NA
No. of miscarriages (mean± SD)	5.6 ± 3.7	4.6 ± 2.0	4.9 ± 2.1	4.9 ± 2.4	NA
No. of miscarriages in a row	4.9 ± 3.4	4.2 ± 1.9	4.5 ± 1.8	4.4 ± 2.2	NA
Children after miscarriage	15 (55.5 %)	82 (86.3 %)	30 (69.8 %)	127 (77.0 %)	NA
No. of pregnancies	6.6 ± 3.9	6.4 ± 2.3	6.2 ± 2.2	6.4 ± 2.6	2.3 ± 0.9

7.1.3 Differential gene expression in endometrium of women with recurrent miscarriage and its' biological relevance

Microarray and IPA

In total, 124 genes were differently expressed in women with recurrent miscarriage, with 30 genes were up- regulated and 94 genes downregulated by a minimum of two-fold in women with recurrent miscarriage compared to fertile controls. The differently expressed shown genes in to groups have been visualized by using a heat map in (Figure 11).

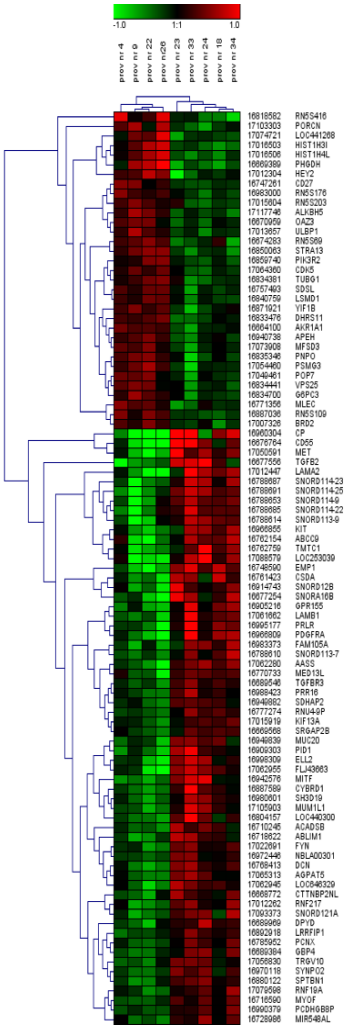

Figure 11. The figure shows Heat map of gene expression in the receptive endometrium from four women with recurrent pregnancy loss and five control women. Green represents down-regulated genes and red represents up-regulated genes.

Of the differently expressed genes in women with recurrent miscarriage, 67 % (19 of 27) were related to immune and inflammatory processes. The IPA analysis showed that the most interesting network was “Reproductive system disease” with IL8 in center of this network (Figure 12). The most up regulated gene was Olfactomedin 4 (OLFM4) and the two most down regulated genes were progestogen-associated endometrial protein (PAEP) and Stanniocalcin 1 (STC1), the latter well known for being related to endometrial receptivity.

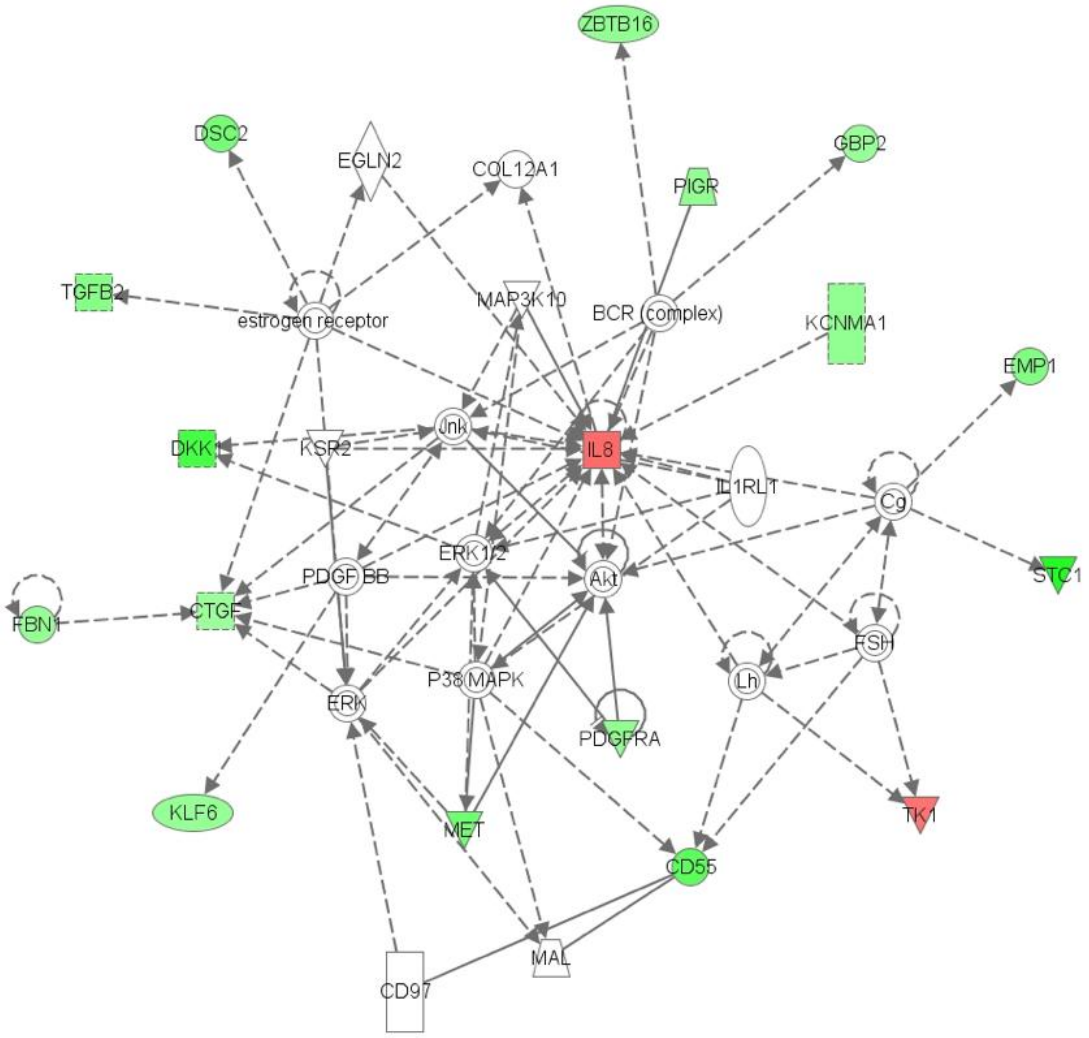

Figure 12 .The highest score after IPA analysis of endometrium from women with recurrent miscarriage compared and from control women was “Reproductive system disease“. Down-regulated genes are indicated as green while up- regulated genes are indicated as red.

7.1.4 Thyroid hormone and thyroid hormone related proteins in endometrium, Fallopian tube and embryo

TSH, fT4 and TPO-Ab in unexplained infertile women compared with fertile controls

Women with unexplained infertility had significantly higher serum levels of fT4. There were no significant differences in the serum levels of TSH and TPO-Ab between the groups.

Protein staining of TSH R, TRα1, TRβ1, DIO2 and MCT8 in endometrium from unexplained infertile women and fertile controls

The highest protein staining of TSH R, TRα1 and TRβ1 was identified in the luminal epithelium. There was significantly lower protein staining of TRα1 and MCT8 in endometrial glandular epithelium from women with unexplained infertility compared to endometrium from fertile controls (Figure 13)

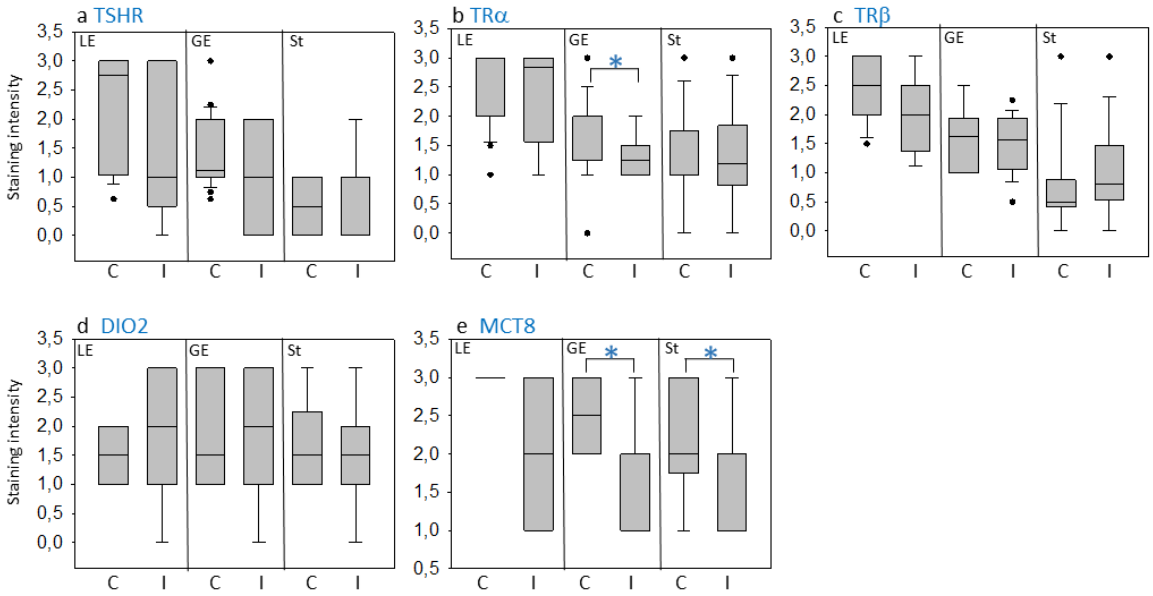

Figure 13. Endometrial protein staining of TSH R (a), TRα1 (b), TRβ1 (c), DIO2 (d) and MCT8 (e) in endometrium from women with unexplained infertility compare to controls. The staining intensity was generally higher in the luminal epithelium (LE) followed by the glandular epithelium (GE) and the least intense staining was seen in the stroma (St). Statistical differences were calculated according to Kruskal-Wallis U-test, $p < 0.05$ was considered statistical difference (*).

Protein staining of TSH R, TRα1, TRβ1 and DIO2 in the Fallopian tube

Staining of proteins related to the thyroid hormone system was seen in the Fallopian tube. The TSH R, TRα1, TRβ1 and DIO2 proteins were identified in the epithelium, muscle and vessels with the highest staining in the epithelium, p <0.001 (Figure 14 and 15). There was no difference between the istmus, ampulla and fimbrie and there was no difference between the follicular and luteal phases of the menstrual cycle.

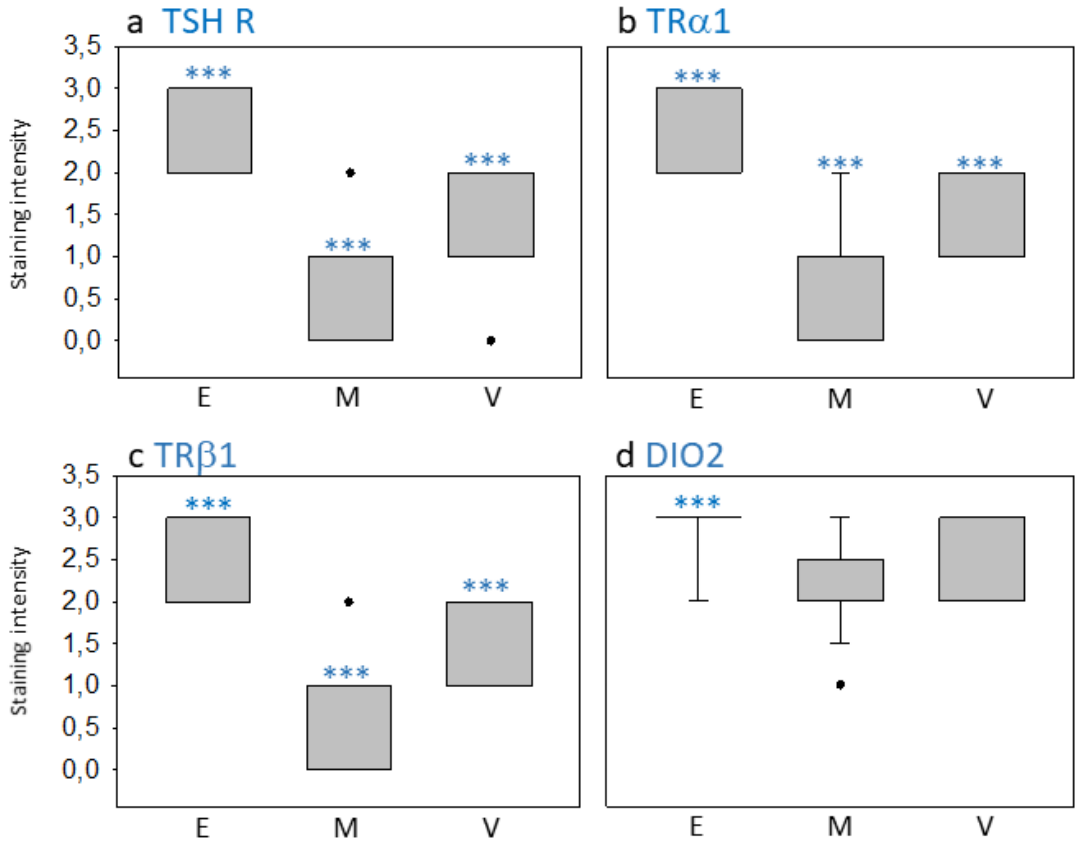

Figure 14. The immune staining intensity of TSH R (a), TRα1 (b), TRβ1 (c) and DIO2 (d) in the three compartments of Fallopian tube from fertile women. The staining intensity of TSH R, TRα1, TRβ1 and DIo2 was significantly different in all three compartments of fallopian tubes; Epithelial cells (E), Vessels (V), Muscle (M). Statistics according to Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn's test, p < 0.001 (***) was considered statistical difference.

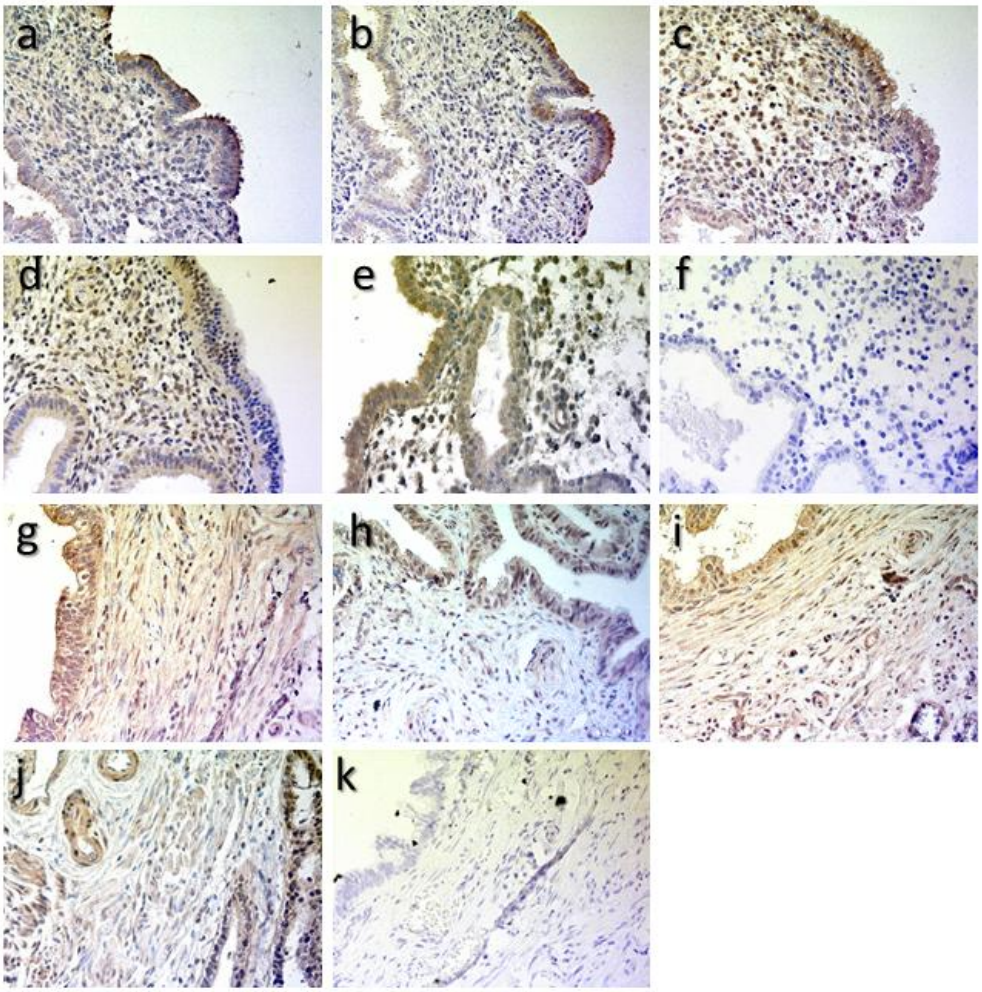

Figure 15. Representative microscopic photos of immunohistochemical staining of a) TSHR, b) TRα, c) TRβ, d) DIO2 and e) MCP8 in endometrium and g) TSHR, h) TRα, i) TRβ and j) DIO2 in Fallopian tube. Negative control was performed by omission of the primary antibody as seen in f) endometrium and k) Fallopian tube. Magnification was 400X.

Thyroid hormone and embryo development

After addition of T4 to the culture media, the number of good quality embryos, was significantly higher in the T4 treated group, 25 (65 %), compared to embryo in control group 18 (50 %, p = 0.031, Figure 16).

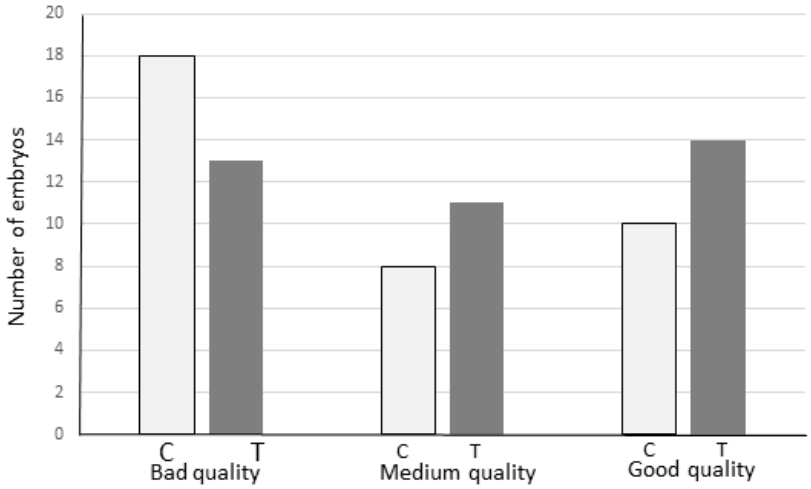

Figure 16. Number of embryos that reached a good quality blastocyst in the control group, (C), and in the thyroid hormone treated group, (T). Statistics according to Fischer's exact test was calculated on the percentage of medium and good quality embryos, $p < 0.05$ was considered statistical difference.

8. DISCUSSION

It is well known that thyroid dysfunction has a negative influence on female fertility and also increasing evidence of a correlation between untreated thyroid dysfunction and adverse pregnancy outcome both for the pregnant woman and the growing fetus [40, 44, 46, 53, 125]. This highlights the importance of a proper screening model for detection of thyroid dysfunction during early pregnancy.

Our study showed almost the same incidence of SCH and hypothyroidism (9.6 % and 10.2 %) in all pregnant women regardless of risk for thyroid disease. This confirms the results of a previous study conducted in Uppsala community in Sweden [126], which showed similar incidences of elevated TSH both in a target screening group (12.1 %), and in an unselected screening group (12.6 %). Furthermore, a Cochrane review of different screening methods for TSH during early pregnancy showed that the number of women diagnosed and subsequently treated for thyroid dysfunction is increased after general screening [127].

A few concerns have been raised about treatment with LT4 in a high percentage of pregnant women. One consideration is the cost benefit of treatment since the evidence for treatment is not conclusive. There are three prospective randomized studies on this aspect; only two studies have evaluated the impact of treatment on fetal neurodevelopment. None of them showed any significant improvement in IQ in the children. However, none of these studies has evaluated the impact of treatment during the first trimester of pregnancy. [47, 128, 129]. The fact that several previous observational and retroactive studies have shown a correlation between SCH and adverse maternal and fetal complications highlights the complexity of this context. [46, 130-132]

One other consideration is the possible adverse effect of LT4 treatment on the developing fetus. The risk of treatment with LT4 during pregnancy has been considered minimal [133]. A study based on the Swedish Birth Register showed a slightly higher risk for congenital malformations, anal and anorectal atresia, in children born to women on LT4 supplementation. However, women on LT4 treatment showed a higher incidence of other diseases such as cardiovascular disease and psychological diagnosis like depression. The Malformations in these categories may have association with other treatments or the underlying diseases, including hypothyroidism. [134].

Moreover, our study highlights the importance of early adjustment of therapy in women with hypothyroidism on LT4 treatment. In 50 % of cases, the women on LT4 treatment had

suboptimal LT4 treatment. This is in agreement with previous studies by Hallengren et al. and Vadiveloo et al., who reported that 30 % of women on LT4 treatment had suboptimal treatment [135, 136]. The fetus is totally dependent on maternal thyroid hormone during first trimester of pregnancy. Thus, it is of importance to increase doses of LT4 as soon as the pregnancy is confirmed [137].

There is an ongoing debate on normal upper limit of TSH during pregnancy and benefits of thyroxine therapy in women with SCH. The profound change in thyroid physiology during pregnancy requires different reference values for fT4 and TSH [51]. International guidelines have recommended assessment of trimester specific population-based reference ranges [138]. In the absence of such reference values, use of international trimester specific cut-off levels is recommended [133].

In our study, the use of TSH 2.0 mIU/L as cut-off level was based on local clinical guidelines for treatment of LT4 at the time of the study. In 2017, the previous recommendation for the upper limit of serum TSH levels during the first trimester was changed from TSH 2.5 mIU/L to 3.5 mIU/L by the American Thyroid Association [133]. However, a cut-off level of TSH < 2.5 mIU/L has been recommended for women with overt hypothyroidism on LT4 supplementation [133]. The Swedish guidelines for treatment of SCH during pregnancy now include an upper limit of TSH at 3.5 mIU/L during the first trimester and general screening during early pregnancy has been recommended (http://online.liebertpub.com/doi/pdfplus/10.1089/thy.2016.0457).

An association between polymorphism in *HABP2* gene with unexplained infertility has been observed in a previous study [102]. Since HABP2 protein in endometrial epithelial cell is up regulated during the mid-secretory phase, it has been identified as a putative biomarker of receptive endometrium [91]. Therefore, as it can be assumed that recurrent miscarriage might be related to endometrial receptivity failure, the association between recurrent miscarriage and polymorphism in *HABP2* gene was studied. The three studied SNPs in the *HABP2* gene were similar in women with recurrent miscarriage and controls. However, we also observed that a polymorphism in the promotor region, rs1157916, was more frequent among women with recurrent miscarriage than in the control group.

Furthermore, this study showed that women with recurrent miscarriage have a high chance of successful pregnancy, almost 80%, but that the number of previous miscarriages, five or more, had a negative impact on the live birth rate, which is in line with the results of other studies.[139, 140]. The limitation of our study was a relative short follow-up times, two

years, that may influence the results. Therefore, additional studies are required to clarify the relationship between HABP2 and recurrent miscarriage.

In a comparison of almost 30 000 genes expressed in receptive endometrium between women with recurrent miscarriage and fertile controls, a different expression pattern was seen. In total, 124 genes were expressed differently in women with recurrent miscarriage. Functional analysis of these genes revealed that most of the dysregulated genes were involved in immune response and inflammatory processes. Particularly, the inflammatory mediator, IL8 gene expression was up regulated in women with recurrent miscarriage. Inflammatory mediators play an important role in implantation, probably by involvement in the selection of a good quality embryo [141]

The highest difference was seen for OLFM4, STC1 and PAEP. These proteins are known to be associated with implantation. OLFM4 is an estrogen regulated protein and seems to be involved in tissue remodeling of the endometrium before implantation. PAEP has suppressive effects of trophoblast activity during implantation and can also modulate trophoblast activity. [142].

Although the majority of early miscarriages is associated with abnormal embryo karyotype, women with many miscarriages have high frequency of embryos with normal karyotype [143]. It has been suggested that an impaired endometrial decidualization in women with recurrent miscarriage facilitates implantation of embryos of poor quality that subsequently leads to miscarriage [144].

In the present study, women with unexplained infertility showed a slightly higher level of serum fT4 and reduced protein staining of TRα1 and MCT8. The lower levels of TRα1 in endometrium from infertile women might require higher levels of fT4. It has been shown that thyroid hormone supplementation in rats increase the expression of TRα and TRβ in the uterus [145].

In addition, lower levels of MCT8 were found in both endometrial glandular epithelial and stromal cells in infertile women. The action of thyroid hormone is dependent on intra-cellular transport (ref). The reduced levels of TRα1 might be a result of low intracellular levels due to reduced levels of transport proteins.

Furthermore, the present study showed higher levels of MCT8 protein during the mid-luteal phase, which indicates that intracellular transport of TH into the epithelial cells might be important for normal receptivity of the endometrium.

The presences of different proteins that are involved in the direct actions of the thyroid system on the Fallopian tubes in combination with enhanced embryo development under T4 substitution, indicate a physiological role of thyroid hormone during early embryo development. Furthermore, in a systematic review, it was shown that sufficient treatment with LT4 in women undergoing IVF/ICSI improved of take home baby rate. Therefore, LT4 supplementation for women with SCH and/or thyroid autoimmunity who are undergoing fertility treatment is needed [146].

9. SUMMARY AND CONCLUSIONS

SUMMARY

- High-risk screening is not optimal to determine which women are at risk of thyroid disease during early pregnancy. Sufficient treatment with LT4 during early pregnancy is important in women with known hypothyroidism.
- There was no association between SNPs in the *HABP2* gene and recurrent miscarriages. However, women with recurrent miscarriage have good prognosis for subsequent live birth.
- Differently expressed endometrial genes in women with recurrent miscarriages emphasize the importance of normal development into a receptive endometrium, required for normal embryo implantation.
- The Thyroid system seems to be imbalanced in women with unexplained infertility.
- The presence of TRα1, TRβ1 and TSH R, and the thyroid hormone related proteins, MCT8 and D2 in the Fallopian tubes in combination with improvement of embryo development by T4 treatment show that thyroid hormones are important for early embryo development.

CONCLUSIONS

Adequate diagnosis and treatment of thyroid disease in women that are either pregnant or trying to become pregnant is of importance for successful pregnancy outcome.

It is likely that recurrent miscarriage is associated with aberrant inflammatory response rather than polymorphism in the *HABP2* gene.

10. FUTURE PERSPECTIVE

- To establish a trimester specific population based TSH reference range in Stockholm.
- To clarify possible genetic associations with recurrent miscarriage, recently developed methods like whole genome sequencing would assist in clarifying this.
- The exact mechanisms behind thyroid disturbances and fertility problems are still not known and further studies on thyroid-related proteins and the reproductive system is needed.

11. SAMMANFATTNING PÅ SVENSKA

Oförklarad barnlöshet drabbar 10-15 % av alla par, som vill ha barn. Upprepade missfall, (fler än 3), inträffar hos 1 till 3 % av alla gravida kvinnor och orsaken är okänd hos 50 %. Rubbningar i sköldkörtelfunktionen kan orsaka barnlöshetsproblem hos kvinnor.

Avsikten med den här avhandlingen var att studera sköldkörtelfunktionens och livmoderslemhinnans betydelse för implantation och graviditet och effekten av sköldkörtelhormon på human embryoutveckling.

Förekomsten av rubbningar av sköldkörtelfunktionen studerades i tre grupper av kvinnor i tidig graviditet. En hög risk, en låg risk och en normal risk för att utveckla underfunktion i sköldkörteln. Subklinisk underfunktion förelåg, då TSH-nivåerna var högre än 2,5 mIE/L. Det förelåg ingen skillnad mellan grupperna.

Kvinnor med upprepade missfall och kontroller, inkluderades i dels i en genetisk analyser i *HABP2*-genen och dels genom att studera genuttryck i livmoderslemhinnan. Inga skillnader i *HABP2*-genen kunde påvisas mellan grupperna. Totalt 124 gener uttrycktes olika i livmoderslemhinna från kvinnor med upprepade missfall jämfört med kontroller. Dessa gener var huvudsakligen involverade i immunologiska processer, speciellt genom uppreglering av IL8.

Hos kvinnor med oförklarad barnlöshet och kontroller studerades sköldkörtel relaterade proteiner i livmoderslemhinnan och äggledarna. Kvinnor med oförklarad barnlöshet hade lägre nivåer av TRα1 och MCT 8 i livmoderslemhinnan än kontrollerna. Man kunde även se infärgning av färgning av sköldkörtelrelaterade proteiner i äggledarna.

Humana embryon användes för att studera effekterna på utvecklingen av embryot. En grupp embryon behandlades med sköldkörtelhormon och den andra gruppen var kontroller. I behandlingsgruppen såg man en förbättrad embryoutveckling jämfört med de obehandlade kontrollerna.

Sammanfattningsvis: En allmän screening av sköldkörtel funktionen av alla kvinnor i tidig graviditet bör införas, (vilket numera är infört i Sverige),

Det krävs fler och större studie för att kartlägga sköldkörtelns betydelse för fruktsamhet, graviditet och embryo utveckling.

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13. REFERENCES

1. Werner, S.C. and J.A. Nauman, *The thyroid.* Annu Rev Physiol, 1968. **30**: p. 213-44.
2. Farid, N.R. and M.W. Szkudlinski, *Minireview: structural and functional evolution of the thyrotropin receptor.* Endocrinology, 2004. **145**(9): p. 4048-57.
3. Vassart, G., L. Pardo, and S. Costagliola, *A molecular dissection of the glycoprotein hormone receptors.* Trends Biochem Sci, 2004. **29**(3): p. 119-26.
4. Aghajanova, L., et al., *Thyroid-stimulating hormone receptor and thyroid hormone receptors are involved in human endometrial physiology.* Fertil Steril, 2011. **95**(1): p. 230-7, 237 e1-2.
5. Aghajanova, L., et al., *Receptors for thyroid-stimulating hormone and thyroid hormones in human ovarian tissue.* Reprod Biomed Online, 2009. **18**(3): p. 337-47.
6. Schweizer, U., J.M. Weitzel, and L. Schomburg, *Think globally: act locally. New insights into the local regulation of thyroid hormone availability challenge long accepted dogmas.* Mol Cell Endocrinol, 2008. **289**(1-2): p. 1-9.
7. Gereben, B., et al., *Cellular and molecular basis of deiodinase-regulated thyroid hormone signaling.* Endocr Rev, 2008. **29**(7): p. 898-938.
8. Friesema, E.C., et al., *Thyroid hormone transporters.* Vitam Horm, 2005. **70**: p. 137-67.
9. Flamant, F., et al., *International Union of Pharmacology. LIX. The pharmacology and classification of the nuclear receptor superfamily: thyroid hormone receptors.* Pharmacol Rev, 2006. **58**(4): p. 705-11.
10. Cheng, S.Y., J.L. Leonard, and P.J. Davis, *Molecular aspects of thyroid hormone actions.* Endocr Rev, 2010. **31**(2): p. 139-70.
11. Williams, G.R., *Cloning and characterization of two novel thyroid hormone receptor beta isoforms.* Mol Cell Biol, 2000. **20**(22): p. 8329-42.
12. O'Shea, P.J., et al., *Characterization of skeletal phenotypes of TRalpha1 and TRbeta mutant mice: implications for tissue thyroid status and T3 target gene expression.* Nucl Recept Signal, 2006. **4**: p. e011.
13. Vanderpump, M.P., et al., *The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham Survey.* Clin Endocrinol (Oxf), 1995. **43**(1): p. 55-68.
14. Andersson, M., V. Karumbunathan, and M.B. Zimmermann, *Global iodine status in 2011 and trends over the past decade.* J Nutr, 2012. **142**(4): p. 744-50.
15. Zimmermann, M.B. and M. Andersson, *Update on iodine status worldwide.* Curr Opin Endocrinol Diabetes Obes, 2012. **19**(5): p. 382-7.
16. McLeod, D.S. and D.S. Cooper, *The incidence and prevalence of thyroid autoimmunity.* Endocrine, 2012. **42**(2): p. 252-65.

17. Poppe, K. and D. Glinoer, *Thyroid autoimmunity and hypothyroidism before and during pregnancy.* Hum Reprod Update, 2003. **9**(2): p. 149-61.

18. Jacobson, D.L., et al., *Epidemiology and estimated population burden of selected autoimmune diseases in the United States.* Clin Immunol Immunopathol, 1997. **84**(3): p. 223-43.

19. Huber, G., et al., *Prospective study of the spontaneous course of subclinical hypothyroidism: prognostic value of thyrotropin, thyroid reserve, and thyroid antibodies.* J Clin Endocrinol Metab, 2002. **87**(7): p. 3221-6.

20. Premawardhana, L.D., et al., *Thyroid peroxidase antibodies in early pregnancy: utility for prediction of postpartum thyroid dysfunction and implications for screening.* Thyroid, 2004. **14**(8): p. 610-5.

21. Mandel, S.J., *Hypothyroidism and chronic autoimmune thyroiditis in the pregnant state: maternal aspects.* Best Pract Res Clin Endocrinol Metab, 2004. **18**(2): p. 213-24.

22. Gordon, G.G. and A.L. Southren, *Thyroid - hormone effects on steroid - hormone metabolism.* Bull N Y Acad Med, 1977. **53**(3): p. 241-59.

23. Krassas, G.E., et al., *Disturbances of menstruation in hypothyroidism.* Clin Endocrinol (Oxf), 1999. **50**(5): p. 655-9.

24. Akinci, B., A. Comlekci, and M.A. Ozcan, *The alteration of coagulation in patients with thyroid dysfunction.* Recent Pat Endocr Metab Immune Drug Discov, 2011. **5**(1): p. 50-7.

25. Simone, J.V., C.F. Abildgaard, and I. Schulman, *Blood coagulation in thyroid dysfunction.* N Engl J Med, 1965. **273**(20): p. 1057-61.

26. Poppe, K., et al., *Thyroid dysfunction and autoimmunity in infertile women.* Thyroid, 2002. **12**(11): p. 997-1001.

27. Akande, E.O. and T.D. Hockaday, *Plasma oestrogen and luteinizing hormone concentrations in thyrotoxic menstrual disturbance.* Proc R Soc Med, 1972. **65**(9): p. 789-90.

28. Redmond, G.P., *Thyroid dysfunction and women's reproductive health.* Thyroid, 2004. **14 Suppl 1**: p. S5-15.

29. Skjoldebrand, L., et al., *Thyroid associated components in serum during normal pregnancy.* Acta Endocrinol (Copenh), 1982. **100**(4): p. 504-11.

30. Laurell, C.B. and G. Rannevik, *A comparison of plasma protein changes induced by danazol, pregnancy, and estrogens.* J Clin Endocrinol Metab, 1979. **49**(5): p. 719-25.

31. Dafnis, E. and S. Sabatini, *The effect of pregnancy on renal function: physiology and pathophysiology.* Am J Med Sci, 1992. **303**(3): p. 184-205.

32. Davison, J.M., *The kidney in pregnancy: a review.* J R Soc Med, 1983. **76**(6): p. 485-501.

33. Glinoer, D., et al., *A randomized trial for the treatment of mild iodine deficiency during pregnancy: maternal and neonatal effects.* J Clin Endocrinol Metab, 1995. **80**(1): p. 258-69.

34. Yoshimura, M., et al., *Mechanism of thyroid stimulation by human chorionic gonadotropin in sera of normal pregnant women.* Acta Endocrinol (Copenh), 1991. **124**(2): p. 173-8.

35. Morreale de Escobar, G., et al., *Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function.* Endocrinology, 1985. **117**(5): p. 1890-900.

36. Contempre, B., et al., *Detection of thyroid hormones in human embryonic cavities during the first trimester of pregnancy.* J Clin Endocrinol Metab, 1993. **77**(6): p. 1719-22.

37. Bernal, J., *Thyroid Hormones in Brain Development and Function*, in *Endotext*, L.J. De Groot, et al., Editors. 2000, MDText.com, Inc.: South Dartmouth (MA).

38. Zoeller, R.T. and J. Rovet, *Timing of thyroid hormone action in the developing brain: clinical observations and experimental findings.* J Neuroendocrinol, 2004. **16**(10): p. 809-18.

39. Stagnaro-Green, A., et al., *The thyroid and pregnancy: a novel risk factor for very preterm delivery.* Thyroid, 2005. **15**(4): p. 351-7.

40. Krassas, G., S.N. Karras, and N. Pontikides, *Thyroid diseases during pregnancy: a number of important issues.* Hormones (Athens), 2015. **14**(1): p. 59-69.

41. Plowden, T.C., et al., *Subclinical Hypothyroidism and Thyroid Autoimmunity Are Not Associated With Fecundity, Pregnancy Loss, or Live Birth.* J Clin Endocrinol Metab, 2016. **101**(6): p. 2358-65.

42. Oostenbroek, M.H.W., et al., *Maternal hypothyroxinaemia in early pregnancy and problem behavior in 5-year-old offspring.* Psychoneuroendocrinology, 2017. **81**: p. 29-35.

43. Moog, N.K., et al., *Influence of maternal thyroid hormones during gestation on fetal brain development.* Neuroscience, 2017. **342**: p. 68-100.

44. Pop, V.J., et al., *Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study.* Clin Endocrinol (Oxf), 2003. **59**(3): p. 282-8.

45. Modesto, T., et al., *Maternal Mild Thyroid Hormone Insufficiency in Early Pregnancy and Attention-Deficit/Hyperactivity Disorder Symptoms in Children.* JAMA Pediatr, 2015. **169**(9): p. 838-45.

46. Haddow, J.E., et al., *Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child.* N Engl J Med, 1999. **341**(8): p. 549-55.

47. Casey, B.M. and E.A. Thom, *Subclinical Hypothyroidism or Hypothyroxinemia in Pregnancy.* N Engl J Med, 2017. **377**(7): p. 701.

48. Smith, C., et al., *Congenital thyrotoxicosis in premature infants.* Clin Endocrinol (Oxf), 2001. **54**(3): p. 371-6.

49. Weetman, A.P., *Immunity, thyroid function and pregnancy: molecular mechanisms.* Nat Rev Endocrinol, 2010. **6**(6): p. 311-8.

50. Goodwin, T.M., et al., *The role of chorionic gonadotropin in transient hyperthyroidism of hyperemesis gravidarum.* J Clin Endocrinol Metab, 1992. **75**(5): p. 1333-7.

51. Glinoer, D., et al., *Regulation of maternal thyroid during pregnancy.* J Clin Endocrinol Metab, 1990. **71**(2): p. 276-87.

52. Dashe, J.S., et al., *Thyroid-stimulating hormone in singleton and twin pregnancy: importance of gestational age-specific reference ranges.* Obstet Gynecol, 2005. **106**(4): p. 753-7.

53. Krassas, G.E., K. Poppe, and D. Glinoer, *Thyroid function and human reproductive health.* Endocr Rev, 2010. **31**(5): p. 702-55.

54. Lazarus, J.H., *Thyroid function in pregnancy.* Br Med Bull, 2011. **97**: p. 137-48.

55. Dunn, C.L., R.W. Kelly, and H.O. Critchley, *Decidualization of the human endometrial stromal cell: an enigmatic transformation.* Reprod Biomed Online, 2003. **7**(2): p. 151-61.

56. Tranguch, S., et al., *Molecular complexity in establishing uterine receptivity and implantation.* Cell Mol Life Sci, 2005. **62**(17): p. 1964-73.

57. Noyes, R.W., A.T. Hertig, and J. Rock, *Dating the endometrial biopsy.* Am J Obstet Gynecol, 1975. **122**(2): p. 262-3.

58. Cornelius, D.C. and K. Wallace, *Decidual natural killer cells: A critical pregnancy mediator altered in preeclampsia.* EBioMedicine, 2019. **39**: p. 31-32.

59. Girling, J.E. and P.A. Rogers, *Regulation of endometrial vascular remodelling: role of the vascular endothelial growth factor family and the angiopoietin-TIE signalling system.* Reproduction, 2009. **138**(6): p. 883-93.

60. Girling, J.E. and P.A. Rogers, *Recent advances in endometrial angiogenesis research.* Angiogenesis, 2005. **8**(2): p. 89-99.

61. Ezzati, M., et al., *Tubal transport of gametes and embryos: a review of physiology and pathophysiology.* J Assist Reprod Genet, 2014. **31**(10): p. 1337-47.

62. Faddy, M.J., et al., *Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause.* Hum Reprod, 1992. **7**(10): p. 1342-6.

63. Gougeon, A., *Dynamics of follicular growth in the human: a model from preliminary results.* Hum Reprod, 1986. **1**(2): p. 81-7.

64. McGee, E.A. and A.J. Hsueh, *Initial and cyclic recruitment of ovarian follicles.* Endocr Rev, 2000. **21**(2): p. 200-14.

65. Nippoldt, T.B., et al., *The roles of estradiol and progesterone in decreasing luteinizing hormone pulse frequency in the luteal phase of the menstrual cycle.* J Clin Endocrinol Metab, 1989. **69**(1): p. 67-76.

66. Kumar, T.R., et al., *Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.* Nat Genet, 1997. **15**(2): p. 201-4.

67. Miro, F. and L.J. Aspinall, *The onset of the initial rise in follicle-stimulating hormone during the human menstrual cycle.* Hum Reprod, 2005. **20**(1): p. 96-100.

68. Gougeon, A., *Some aspects of the dynamics of ovarian follicular growth in the human.* Acta Eur Fertil, 1989. **20**(4): p. 185-92.

69. Burger, H.G., et al., *Early follicular phase serum FSH as a function of age: the roles of inhibin B, inhibin A and estradiol.* Climacteric, 2000. **3**(1): p. 17-24.

70. La Marca, A., et al., *Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART).* Hum Reprod Update, 2010. **16**(2): p. 113-30.

71. Jabbour, H.N., et al., *Endocrine regulation of menstruation.* Endocr Rev, 2006. **27**(1): p. 17-46.

72. More, I.A., et al., *Cyclical changes in the ultrastructure of the normal human endometrial stromal cell.* J Obstet Gynaecol Br Commonw, 1974. **81**(5): p. 337-47.

73. Rogers, P.A., et al., *Endometrial angiogenesis, vascular maturation, and lymphangiogenesis.* Reprod Sci, 2009. **16**(2): p. 147-51.

74. Choudhury, R.H., et al., *Extravillous Trophoblast and Endothelial Cell Crosstalk Mediates Leukocyte Infiltration to the Early Remodeling Decidual Spiral Arteriole Wall.* J Immunol, 2017. **198**(10): p. 4115-4128.

75. Gaide Chevronnay, H.P., et al., *Regulation of matrix metalloproteinases activity studied in human endometrium as a paradigm of cyclic tissue breakdown and regeneration.* Biochim Biophys Acta, 2012. **1824**(1): p. 146-56.

76. Macklon, N.S., J.P. Geraedts, and B.C. Fauser, *Conception to ongoing pregnancy: the 'black box' of early pregnancy loss.* Hum Reprod Update, 2002. **8**(4): p. 333-43.

77. Nayak, N.R., et al., *Progesterone withdrawal up-regulates vascular endothelial growth factor receptor type 2 in the superficial zone stroma of the human and macaque endometrium: potential relevance to menstruation.* J Clin Endocrinol Metab, 2000. **85**(9): p. 3442-52.

78. Genbacev, O.D., et al., *Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface.* Science, 2003. **299**(5605): p. 405-8.

79. Li, S. and W. Winuthayanon, *Oviduct: roles in fertilization and early embryo development.* J Endocrinol, 2017. **232**(1): p. R1-r26.

80. Salamonsen, L.A., et al., *The Microenvironment of Human Implantation: Determinant of Reproductive Success.* Am J Reprod Immunol, 2016. **75**(3): p. 218-25.

81. Croxatto, H.B., et al., *Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak.* Am J Obstet Gynecol, 1978. **132**(6): p. 629-34.

82. Schlafke, S. and A.C. Enders, *Cellular basis of interaction between trophoblast and uterus at implantation.* Biol Reprod, 1975. **12**(1): p. 41-65.

83. Carson, D.D., et al., *Embryo implantation.* Dev Biol, 2000. **223**(2): p. 217-37.

84. Bonduelle, M.L., et al., *Chorionic gonadotrophin-beta mRNA, a trophoblast marker, is expressed in human 8-cell embryos derived from tripronucleate zygotes.* Hum Reprod, 1988. **3**(7): p. 909-14.

85. Sharma, S., G. Godbole, and D. Modi, *Decidual Control of Trophoblast Invasion.* Am J Reprod Immunol, 2016. **75**(3): p. 341-50.

86. Wegmann, T.G., et al., *Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?* Immunol Today, 1993. **14**(7): p. 353-6.

87. Lopata, A. and D.L. Hay, *The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture.* Hum Reprod, 1989. **4**(8 Suppl): p. 87-94.

88. O'Rahilly, R. and F. Muller, *Developmental stages in human embryos: revised and new measurements.* Cells Tissues Organs, 2010. **192**(2): p. 73-84.

89. Hilton, D.J., *LIF: lots of interesting functions.* Trends Biochem Sci, 1992. **17**(2): p. 72-6.

90. Dimitriadis, E., et al., *Cytokines, chemokines and growth factors in endometrium related to implantation.* Hum Reprod Update, 2005. **11**(6): p. 613-30.

91. Altmae, S., et al., *Meta-signature of human endometrial receptivity: a meta-analysis and validation study of transcriptomic biomarkers.* Sci Rep, 2017. **7**(1): p. 10077.

92. Altmae, S., et al., *Research resource: interactome of human embryo implantation: identification of gene expression pathways, regulation, and integrated regulatory networks.* Mol Endocrinol, 2012. **26**(1): p. 203-17.

93. Lim, K.J., et al., *Profile of cytokine mRNA expression in peri-implantation human endometrium.* Mol Hum Reprod, 1998. **4**(1): p. 77-81.

94. Sato, Y., et al., *Trophoblasts acquire a chemokine receptor, CCR1, as they differentiate towards invasive phenotype.* Development, 2003. **130**(22): p. 5519-32.

95. Salamonsen, L.A., N.J. Hannan, and E. Dimitriadis, *Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation.* Semin Reprod Med, 2007. **25**(6): p. 437-44.

96. Sojka, D.K., L. Yang, and W.M. Yokoyama, *Uterine natural killer cells: To protect and to nurture.* Birth Defects Res, 2018. **110**(20): p. 1531-1538.

97. Ramhorst, R., et al., *Decoding the chemokine network that links leukocytes with decidual cells and the trophoblast during early implantation.* Cell Adh Migr, 2016. **10**(1-2): p. 197-207.

98. Yang, Y., et al., *Association between maternal, fetal and paternal MTHFR gene C677T and A1298C polymorphisms and risk of recurrent pregnancy loss: a comprehensive evaluation.* Arch Gynecol Obstet, 2016. **293**(6): p. 1197-211.

99. Li, H.H., et al., *Association of TNF-alpha genetic polymorphisms with recurrent pregnancy loss risk: a systematic review and meta-analysis.* Reprod Biol Endocrinol, 2016. **14**: p. 6.

100. Karatas, A., et al., *Endothelial nitric oxide synthase gene polymorphisms (promoter -786T/C, exon 894 G/T and intron G10T) in unexplained female infertility.* Gynecol Obstet Invest, 2014. **77**(2): p. 89-93.

101. Ebersberger, I., et al., *Genomewide comparison of DNA sequences between humans and chimpanzees.* Am J Hum Genet, 2002. **70**(6): p. 1490-7.

102. Altmae, S., et al., *Variation in hyaluronan-binding protein 2 (HABP2) promoter region is associated with unexplained female infertility.* Reprod Sci, 2011. **18**(5): p. 485-92.

103. Trompet, S., et al., *Factor VII Activating Protease Polymorphism (G534E) Is Associated with Increased Risk for Stroke and Mortality.* Stroke Res Treat, 2011. **2011**: p. 424759.

104. Etscheid, M., et al., *The Marburg I polymorphism of factor VII activating protease is associated with low proteolytic and low pro-coagulant activity.* Thromb Res, 2012. **130**(6): p. 935-41.

105. Romisch, J., et al., *The FVII activating protease cleaves single-chain plasminogen activators.* Haemostasis, 1999. **29**(5): p. 292-9.

106. Choi-Miura, N.H., et al., *Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator.* J Biochem, 1996. **119**(6): p. 1157-65.

107. Salustri, A., et al., *Hyaluronan and proteoglycans in ovarian follicles.* Hum Reprod Update, 1999. **5**(4): p. 293-301.

108. Salamonsen, L.A., S. Shuster, and R. Stern, *Distribution of hyaluronan in human endometrium across the menstrual cycle. Implications for implantation and menstruation.* Cell Tissue Res, 2001. **306**(2): p. 335-40.

109. Bersinger, N.A., et al., *Gene expression in cultured endometrium from women with different outcomes following IVF*. Mol Hum Reprod, 2008. **14**(8): p. 475-84.

110. Aghajanova, L., et al., *Obstetrics and Gynecology Residency and Fertility Needs.* Reprod Sci, 2017. **24**(3): p. 428-434.

111. Dyer, S.J., *International estimates on infertility prevalence and treatment seeking: potential need and demand for medical care.* Hum Reprod, 2009. **24**(9): p. 2379-80; author reply 2380-3.

112. Baldur-Felskov, B., et al., *Psychiatric disorders in women with fertility problems: results from a large Danish register-based cohort study.* Hum Reprod, 2013. **28**(3): p. 683-90.

113. Barbieri, R.L., *The initial fertility consultation: recommendations concerning cigarette smoking, body mass index, and alcohol and caffeine consumption.* Am J Obstet Gynecol, 2001. **185**(5): p. 1168-73.

114. Barratt, C.L.R., et al., *The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities.* Hum Reprod Update, 2017. **23**(6): p. 660-680.

115. *[Laboratory manual of the WHO for the examination of human semen and sperm-cervical mucus interaction]*. Ann Ist Super Sanita, 2001. **37**(1): p. I-xii, 1-123.

116. *Diagnostic evaluation of the infertile female: a committee opinion.* Fertil Steril, 2015. **103**(6): p. e44-50.

117. Athaullah, N., M. Proctor, and N.P. Johnson, *Oral versus injectable ovulation induction agents for unexplained subfertility.* Cochrane Database Syst Rev, 2002(3): p. Cd003052.

118. Diamond, M.P., et al., *Letrozole, Gonadotropin, or Clomiphene for Unexplained Infertility.* N Engl J Med, 2015. **373**(13): p. 1230-40.

119. Zegers-Hochschild, F., et al., *International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009.* Fertil Steril, 2009. **92**(5): p. 1520-4.

120. Mascarenhas, M.N., et al., *National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys.* PLoS Med, 2012. **9**(12): p. e1001356.

121. Rai, R. and L. Regan, *Recurrent miscarriage.* Lancet, 2006. **368**(9535): p. 601-11.

122. Jauniaux, E., et al., *Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage.* Hum Reprod, 2006. **21**(9): p. 2216-22.

123. Egerup, P., et al., *The Effects of Intravenous Immunoglobulins in Women with Recurrent Miscarriages: A Systematic Review of Randomised Trials with Meta-Analyses and Trial Sequential Analyses Including Individual Patient Data.* PLoS One, 2015. **10**(10): p. e0141588.

124. Sturn, A., J. Quackenbush, and Z. Trajanoski, *Genesis: cluster analysis of microarray data.* Bioinformatics, 2002. **18**(1): p. 207-8.

125. Benhadi, N., et al., *Higher maternal TSH levels in pregnancy are associated with increased risk for miscarriage, fetal or neonatal death.* Eur J Endocrinol, 2009. **160**(6): p. 985-91.

126. Granfors, M., et al., *Targeted thyroid testing during pregnancy in clinical practice.* Obstet Gynecol, 2014. **124**(1): p. 10-5.

127. Reid, S.M., et al., *Interventions for clinical and subclinical hypothyroidism pre-pregnancy and during pregnancy.* Cochrane Database Syst Rev, 2013(5): p. Cd007752.

128. Negro, R., et al., *Universal screening versus case finding for detection and treatment of thyroid hormonal dysfunction during pregnancy.* J Clin Endocrinol Metab, 2010. **95**(4): p. 1699-707.

129. Hales, C., et al., *Controlled Antenatal Thyroid Screening II: Effect of Treating Maternal Suboptimal Thyroid Function on Child Cognition.* J Clin Endocrinol Metab, 2018. **103**(4): p. 1583-1591.

130. Negro, R. and A. Stagnaro-Green, *Diagnosis and management of subclinical hypothyroidism in pregnancy.* Bmj, 2014. **349**: p. g4929.

131. Su, P.Y., et al., *Maternal thyroid function in the first twenty weeks of pregnancy and subsequent fetal and infant development: a prospective population-based cohort study in China.* J Clin Endocrinol Metab, 2011. **96**(10): p. 3234-41.

132. Li, Y., et al., *Abnormalities of maternal thyroid function during pregnancy affect neuropsychological development of their children at 25-30 months.* Clin Endocrinol (Oxf), 2010. **72**(6): p. 825-9.

133. Alexander, E.K., et al., *2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum.* Thyroid, 2017. **27**(3): p. 315-389.

134. Kallen, B. and B. Norstedt Wikner, *Maternal hypothyroidism in early pregnancy and infant structural congenital malformations.* J Thyroid Res, 2014. **2014**: p. 160780.

135. Hallengren, B., et al., *Pregnant women on thyroxine substitution are often dysregulated in early pregnancy.* Thyroid, 2009. **19**(4): p. 391-4.

136. Vadiveloo, T., et al., *Thyroid testing in pregnant women with thyroid dysfunction in Tayside, Scotland: the thyroid epidemiology, audit and research study (TEARS).* Clin Endocrinol (Oxf), 2013. **78**(3): p. 466-71.

137. Alexander, E.K., et al., *Timing and magnitude of increases in levothyroxine requirements during pregnancy in women with hypothyroidism.* N Engl J Med, 2004. **351**(3): p. 241-9.

138. Medici, M., et al., *Thyroid function in pregnancy: what is normal?* Clin Chem, 2015. **61**(5): p. 704-13.

139. Blomqvist, L., M. Hellgren, and A. Strandell, *Acetylsalicylic acid does not prevent first-trimester unexplained recurrent pregnancy loss: A randomized controlled trial.* Acta Obstet Gynecol Scand, 2018. **97**(11): p. 1365-1372.

140. Clifford, K., R. Rai, and L. Regan, *Future pregnancy outcome in unexplained recurrent first trimester miscarriage.* Hum Reprod, 1997. **12**(2): p. 387-9.

141. Brosens, J.J., et al., *Uterine selection of human embryos at implantation.* Sci Rep, 2014. **4**: p. 3894.

142. Bastu, E., et al., *Role of Mucin 1 and Glycodelin A in recurrent implantation failure.* Fertil Steril, 2015. **103**(4): p. 1059-1064 e2.

143. Li, T.C., E.M. Tuckerman, and S.M. Laird, *Endometrial factors in recurrent miscarriage.* Hum Reprod Update, 2002. **8**(1): p. 43-52.

144. Macklon, N.S. and J.J. Brosens, *The human endometrium as a sensor of embryo quality.* Biol Reprod, 2014. **91**(4): p. 98.

145. Sayem, A.S.M., et al., *Effects of thyroxine on expression of proteins related to thyroid hormone functions (TR-alpha, TR-beta, RXR and ERK1/2) in uterus during peri-implantation period.* Biomed Pharmacother, 2017. **96**: p. 1016-1021.

146. Rao, M., et al., *Effect of levothyroxine supplementation on pregnancy loss and preterm birth in women with subclinical hypothyroidism and thyroid autoimmunity: a systematic review and meta-analysis.* Hum Reprod Update, 2019. **25**(3): p. 344-361.